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THYROXIN AS A DEPRESSANT OF THE DIVISION RATE OF PARAMECIUM.

By HARRY BEAL TORREY, MATTHEW C. RIDDLE, AND J. L. BRODIE.

(From the Laboratory of Experimental Biology of the University of Oregon Medical School, Portland.)

(Accepted for publication, July 10, 1924.)

I.

It is now 16 years since Nowikoff (1908) accelerated the division rate of *Paramecium* by adding thyroid gland to his cultures. Later, Shumway (1914) reached the same conclusion after a series of more critical and painstaking experiments, and in the following year Budington and Harvey (1915) showed that thyroids from various vertebrates would produce similar results. Still later, Shumway (1917) observed the effect of total thyroid on other processes of *Paramecium*, notably excretion and gastric vacuole formation. From these observations it appeared that thyroid was augmenting processes of dissimilation in so far as these might be indicated by effects on excretion and at the same time processes of assimilation as indicated by effects on the division rate. This striking combination of contrasting functions suggested the possibility that two distinct causes might be operating, both associated with the thyroid substance.

With this suggestion in mind, a series of experiments was begun some 3 years ago by Miss Dora E. Birchard, a student in this laboratory. Her objective was a comparison of the effects of total thyroid and its crystalline derivative thyroxin on *Paramecium*. With her kind permission the following facts are taken from her unpublished manuscript. The culture medium was stock hay infusion containing a mixed infection of bacteria. Thyroid and thyroxin were added as required, the former (Armour and Company's desiccated, containing 0.2 per cent iodine) in suspension (1:600), the latter (Squibb's) in solution (1:20,000) in approximately N/400 NaOH.

The dosage of thyroxin to which *Paramecium* was exposed was thus in terms of iodine about ten times as large as that of thyroid. The difference, however, was not reflected in the behavior of *Paramecium*, except perhaps in connection with the

division rate. The latter was markedly accelerated in the thyroid lines and greatly depressed in the thyroxin lines. The latter results, however, were not satisfactory, owing to an excessive death rate indicating toxic effects demanding further investigation.

In other respects, as summarized below, the effects of thyroid and thyroxin were similar.

1. The individuals from thyroid and thyroxin cultures were on the average shorter and more slender than control individuals. The following figures represent the average length and width obtained from random samples of 100 individuals in each case: from thyroid culture, 0.196 × 0.068 mm., and control, 0.220 × 0.086 mm.; from thyroxin culture, 0.191 × 0.062 mm., and control (in N/400 NaOH) 0.218 × 0.079 mm.

2. Gastric vacuoles were formed more rapidly in both thyroid and thyroxin cultures than in their controls. Using the method devised by Shumway and basing the figures on a count of 100 individuals in each case, as before, the average number of gastric vacuoles formed in 5 minutes was, for the thyroid cultures, 3.87, an increase of 76 per cent over the control figure of 2.19; for the thyroxin culture, 3.88, an increase of 62 per cent over the control figure of 2.39.

Shumway, whose figures do not show so marked an acceleration of gastric vacuole formation, did not regard the effect of thyroid of much significance in this connection.

3. The contractile vacuoles pulsated at rates 75 per cent greater in thyroid and thyroxin cultures than in their controls. The average rates per minute for groups of 100 individuals were: thyroid cultures, 8.11, control, 4.61; thyroxin cultures, 7.19, control, 4.10.

4. Among two groups of 100 individuals each from thyroid and thyroxin lines three contractile vacuoles were found in two of the former, three of the latter.

5. The canals tributary to the contractile vacuoles were observed to be longer and more numerous in both thyroid and thyroxin lines than in the controls. No detailed counts were made.

6. Non-contractile vacuoles were usually present in the individuals from both thyroid and thyroxin lines. In the controls they were only exceptionally present.

II.

With these results before us, the present authors took up the investigation with a modified technique. The use of sodium hydrate as a solvent of thyroxin was temporarily abandoned. Crystals of thyroxin were added directly to the culture medium, in this case 0.025 per cent beef extract, as recommended by Woodruff and Baitsell (1911).

Pure lines were cultured on the slide in 0.2 cc. drops. Experimental lines and their controls were begun with sister individuals in the customary way. The cultures were examined daily and one individual

from each drop was transferred to a similar drop of culture medium freshly prepared, containing a plentiful supply of a mixed bacterial flora and a green monad, both of which were ingested. All slides were kept in similar moist chambers under similar temperature conditions.

The reaction of the beef extract medium after sterilization in test-tubes was distinctly acid. After infection and incubation for 24 hours it acquired a reaction represented by pH 7.0 to 7.6. The slide culture drops were made from these tubes after the latter had been incubated 24 to 48 hours. It was to these alkaline drops that thyroxin crystals were added.

TABLE I.

Pulsations per Minute of the Contractile Vacuoles.

Group.	Diet.	No. of animals measured.	Average rate of pulsation.	Increase in thyroxin cultures.
				per cent
1	Thyroxin.	1	6	50
	Control.	3	4	
2	Thyroxin.	1	9	38.5
	Control.	3	6.5	
3	Thyroxin.	7	11.7	25.8
	Control.	2	9.3	
4	Thyroxin.	4	10.75	22.9
	Control.	4	8.75	
5	Thyroxin.	4	10	0
	Control.	2	10	
6	Thyroxin.	6	5.83	11
	Control.	8	5.25	

That thyroxin thus administered actually affected the behavior of *Paramecium* was clear from the following facts: that in the thyroxin cultures as compared with the controls, the rate of pulsation of the contractile vacuoles was higher (Table I), the number of canals leading into each vacuole greater (Table II), the average number of food vacuoles less (Table III), and the crystals in the endoplasm resembling those that Schewiakoff (1893) regarded as calcium phosphate, fewer (Table IV).

In these tables the individuals in each group were observed within a few minutes of each other under what appeared to be constant

conditions. Different groups were observed at different times under conditions that could not be regarded as constant for all groups. For this reason and because of the variable number of individuals in the several groups, average figures for all groups in each table would not satisfactorily express the facts, even for larger numbers of individuals than those actually observed. Despite the relatively small number of the latter, and the manner of recording them, the differences between experimental and control individuals are sufficiently marked to justify the conclusions drawn.

In these respects, and under a modified technique, thyroxin again resembled total thyroid in its effect on certain metabolic processes. In its effect upon the division rate, however, thyroxin differed unequivocally from the latter. The average daily division rate calculated from observations on eighteen experimental cultures under observation for periods ranging from 7 to 71 days, was 0.8687 per cent in the controls, 0.8205 per cent in the thyroxin cultures. These figures represent a decrease of 5.54 per cent in the division rate in the latter. This difference is too small in itself to establish conclusively a depressing action of thyroxin on the division rate. But it does make clear that the marked accelerations of the division rate customarily observed following the administration of total thyroid can hardly be due to the thyroxin contained therein.

Attention was called to this conclusion and the facts on which it was based in a preliminary report by Riddle and Torrey (1922–23). This conclusion is cited and supported by Woodruff and Swingle (1923), who are convinced from their experiments, that

> ". . . . neither thyroxin (Squibb's) nor commercial desiccated thyroid, or fresh desiccated thyroid of the turtle produce any significant acceleration of the division rate of *Paramecium*.
>
> "Data to the contrary published by previous investigators apparently are attributable chiefly to variations in the bacterial food supply which the different media afforded the *Paramecia*.
>
> "Accordingly, all the evidence from studies on *Paramecium* to the effect that thyroid products accelerate cell anabolism is, we believe, erroneous."

About the same time, Cori (1923) concluded, from experiments on *Paramecium*, that thyroxin accelerates the division rate though to a much smaller degree than thyroid extract, which thus presumably

contained "another active substance besides thyroxin, which accelerates the division rate."[1]

Prior to the appearance of Woodruff and Swingle's paper we had concluded another series of experiments to test further the difference

TABLE II.

Number of Canals Tributary to Each Contractile Vacuole.

Group.	Diet.	No. of animals measured.	Average No. of canals.		Increase in thyroxin cultures.	
			Anterior vacuole.	Posterior vacuole.	Anterior.	Posterior.
					per cent	*per cent*
2	Thyroxin.	1	11	12	37.5	50
	Control.	3	8	8		
3	Thyroxin.	7	10	10	25	25
	Control.	2	8	8		
4	Thyroxin.	4	9.25	9.25	2.8	13.9
	Control.	4	9	8		
5	Thyroxin.	4	9	9	12.6	20
	Control.	2	8	7.5		
6	Thyroxin.	6	9.83	9.66	17.3	17.1
	Control.	8	8.38	8.25		

TABLE III.

Gastric Vacuoles in Endoplasm.

Group.	Diet.	No. of animals examined.	Average No. of food vacuoles.
3	Thyroxin.	7	17.1
	Control.	2	24.5
4	Thyroxin.	4	8.25
	Control.	4	19.25
5	Thyroxin.	4	19.3
	Control.	2	16
6	Thyroxin.	6	10.5
	Control.	8	16.9

shown in our previous work between the effects of thyroid and thyroxin on the division rate of *Paramecium*, and a preliminary report of the results was published by Torrey (1923).

For these experiments, *Paramecium* was cultured on the slide in

[1] Cori (1923), p. 299.

0.2 cc. drops of 0.025 per cent beef extract medium infected with a mixed culture of bacteria containing a minute flagellate. Four experimental lines with four control lines each containing two files, were established in the usual way, each file and its control beginning with sister individuals, and all sixteen files descended from a single individual four generations back. To experimental line I, thyroxin in crystalline form was given; to experimental line II, NaOH strong enough to give an initial concentration of 0.005 N in the culture drops; to experimental line III, 0.01 per cent thyroxin in the solution of NaOH used for II; to experimental line IV, desiccated thyroid in

TABLE IV.

Crystals in Endoplasm.

Group.	Diet.	No. of animals measured.	Average No. of crystals.	Decrease in thyroxin cultures.
				per cent
1	Thyroxin.	1	51	30.4
	Control.	3	73.3	
2	Thyroxin.	1	38	75.1
	Control.	3	153	
3	Thyroxin.	7	55.2	30.6
	Control.	2	72	
4	Thyroxin.	4	64.4	24.3
	Control.	4	85.25	
5	Thyroxin.	4	89.25	10.8
	Control.	2	100	
6	Thyroxin.	6	57	27.3
	Control.	8	78.4	

suspension (1:1000). Estimated on the basis of iodine content, the thyroxin culture drops contained about thirty times as much thyroxin as the thyroid culture drops.

The results of this series of experiments are shown in the accompanying graphs made from original records subsequently lost through an unfortunate accident.

Each graph represents the division rate of the two files of an experimental line (broken line) and the two files of its control (solid line) averaged for 10 day periods. Experimental conditions continued through four such periods for each file. The graphs also show the average division rate for the period immediately preceding the intro-

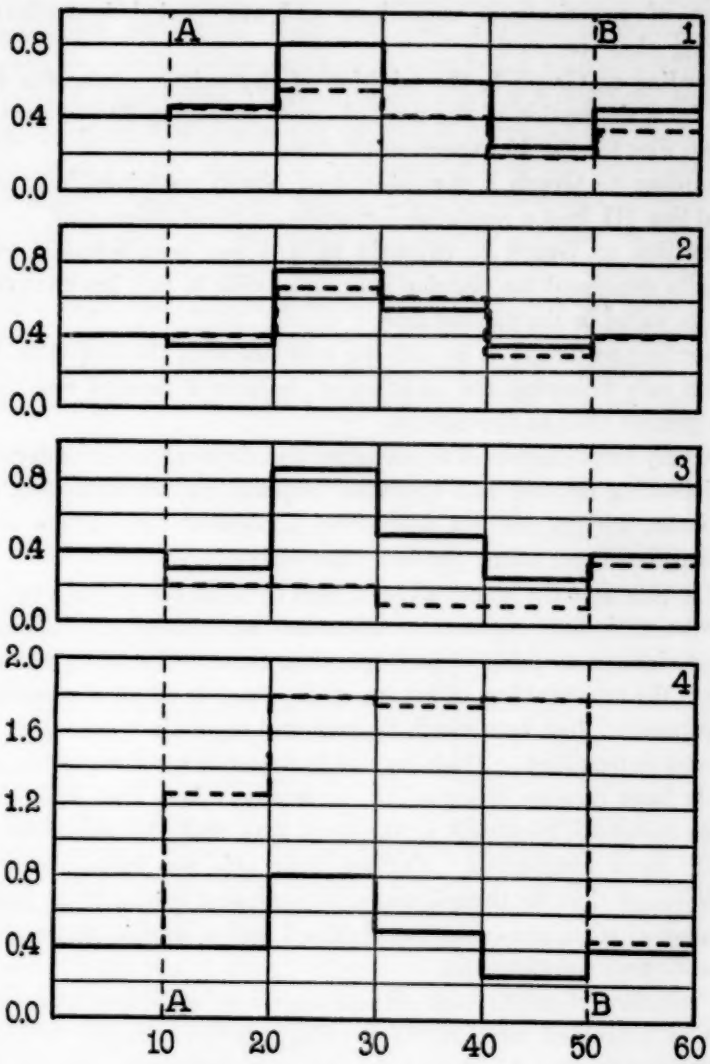

FIG. 1. The division rate of *Paramecium* as affected by total thyroid (marked acceleration) and thyroxin (marked depression). Each graph shows the daily division rate of an experimental culture (broken line) and its control (solid line) averaged for 10 day periods. *A—B* marks the limits of experimental feeding. Graph 1, effect of thyroxin in crystalline form; Graph 2, control for Graph 3; Graph 3, effect of thyroxin in solution; Graph 4, effect of desiccated thyroid.

duction of experimental conditions and the period immediately following their removal.

According to Graph 1, the addition of thyroxin crystals was followed by a depression of the normal curve averaging 20 per cent in the two files for the 40 experimental days.

According to Graph 2, the amount of NaOH contained in experimental line III, had a negligible influence on the division rate.

According to Graph 3, thyroxin in 0.01 per cent solution very markedly depressed the division rate, to a point in fact less than one-third its value in the control line.

According to Graph 4, in striking contrast with the last case, the division rate following the addition of desiccated thyroid was three times greater than in the control.

The very large degrees of acceleration and depression of the division rate following thyroid and thyroxin, respectively, are unique in our experience, and as yet not adequately accounted for. There is no question, however, about the facts themselves.

Up to this time thyroxin had been used by us in concentrations that in terms of iodine content corresponded with doses of desiccated thyroid eighteen to thirty times larger than those of the latter actually given. We are speaking, of course, of the doses to which *Paramecium* was exposed. Just how much thyroid and thyroxin were taken has not been determined. That thyroid is ingested by *Paramecium* has already been directly observed by Shumway. And that it modifies certain metabolic processes in the same way that thyroxin does has already been established. Farther than this we cannot now go except to say that the thyroid was always present in quantities greatly in excess of what the organism obtained and a similar statement is probably true for thyroxin.

In solution, thyroxin was presumably more available than when given in combined form in the thyroid substance itself. This fact would serve to diminish the relative amount of thyroxin administered as thyroid, in comparison with that available in soluble form.

Even though it is probable that thyroxin was present in our drop cultures in amounts far in excess of what *Paramecium* actually absorbed, the degree of its concentration in solution played an appreciable rôle in the determination of the division rate. This was clearly shown in the following experiment.

Four groups of drop cultures, each consisting of twelve files, were begun with *Paramecia* descended from a single individual. Corresponding files in the several groups were begun with granddaughters of the same individual. The culture medium was 0.025 per cent beef extract[2] infected with a mixed culture of bacteria from the air, containing as before the small flagellate previously mentioned. To this control medium were added, for the three experimental lines, 0.01 per cent, 0.001 per cent, and 0.0001 per cent thyroxin, respectively, dissolved in N/2,500 NaOH. The pH figure was taken at the beginning and end of each 24 hour period.[3] The diurnal variation was between 7.4 and 7.6 or 7.7 for all files. On only one day was this latter figure exceeded, when the control medium with an initial pH of 7.7 reached pH 8.0 by the end of the 24 hour day. Each

TABLE V.

Group.		pH diurnal variation.	No. of divisions.	Average daily division rate.	Ratio of experimental division rate to control.
I.	Control (0.025 per cent beef extract).	7.4–7.7 (On 1 day 7.7–8.0)	244	2.033	1.00
II.	Thyroxin 0.0001 per cent.	7.4–7.7	230	1.91	0.942
III.	" 0.001 " "	7.4–7.7	215	1.79	0.881
IV.	" 0.01 " "	7.4–7.7	178	1.483	0.729

[2] Woodruff and Baitsell (1911) reached their standard concentration of beef extract by a process of dilution. This procedure was necessary, as it appears, in order to reduce the acidity of the medium which in higher concentrations is too great for *Paramecium*. At the same time, however, dilution lessened the nutritive value of the medium for the bacteria which serve as food for *Paramecium*. After the data recorded in the text were obtained, we found that the acidity of the medium could be readily controlled by sodium hydroxide without interfering with the superior nutritive values of higher concentrations. *Paramecium* thrives in 0.05 and 0.075 per cent beef extract adjusted to a pH of 7.4 to 7.8.

[3] This was obtained by the drop method described by Felton (1921). Buffer mixtures were made of Sörensen's primary potassium phosphate and secondary sodium phosphate as recommended by Clark (1922). Phenol red, brom-cresol purple, and brom-phenol blue were used as indicators, made up in sodium hydroxide according to his directions. The tests were made commonly on a clear sheet of glass, with neutral surface over a white ground, instead of on a porcelain plate. The indicator was also added directly to the culture drop on the culture side.

file was observed for 10 days, morning and evening, when all save one individual were removed from each drop. The culture medium was changed each morning. All cultures were kept at the same temperature, which varied during the course of the experiment from 24–27°C.

Table V summarizes the results for the 10 day period.

These figures show that thyroxin in solution in a concentration as low as 1 part in 1,000,000 (Group II) depresses the division rate slightly but distinctly, and that thyroxin in the higher concentrations shows more marked effects in the same direction. The magnitude of the effect, however, does not increase with the same rapidity as the concentration of thyroxin. Indeed, the curve of depression flattens rapidly as the concentration of thyroxin increases, reaching a maximum value of 27 per cent as the solution of thyroxin, at 1 part in 10,000, approaches saturation in the concentration of NaOH used. Thus while the division rate is unmistakably depressed in these experiments by this maximum concentration of thyroxin, it is far from completely inhibited.

That the action of thyroxin on *Paramecium* is direct and not by way of an effect on the food supply, as in the case of total thyroid according to Woodruff and Swingle (1923), is supported by the effect of thyroxin on the food organisms in both drop and mass cultures.

Many culture drops containing a superabundance of organisms were stained at the end of 24 hours and compared with respect to the abundance of the organisms as a whole, and the relative abundance of the several kinds involved. Inspection revealed no appreciable differences in either case between control drops and drops containing the several concentrations of thyroxin used in the experiments.

The evidence from mass cultures was obtained by infecting equal amounts of 0.05 per cent beef extract medium corrected to pH 7.4 and containing the concentration of thyroxin (in NaOH) desired, in test-tubes of the same diameter, with equal amounts of a suspension of a given species or mixture of species, incubating for 24 hours, and determining the relative turbidity of the medium at the end of that time. The tubes were infected, in lots of several each, (1) with the mixed culture just mentioned, (2) with a pure culture of *Bacillus subtilis*, and (3) with a pure culture of *Bacillus fluorescens*. Thyroxin was used in concentrations of 1:10,000, 1:100,000, and 1:1,000,000.

From the first no satisfactory test was obtained owing to the tendency of thyroxin at this concentration to precipitate out of solution, increasing the turbidity thereby beyond the degree reached in the other tubes.

At the end of 24 hours, good growths were observed in all the tubes. The mixed cultures tended to flock, owing to the presence of a non-motile form which grew in sheets. The cultures of both pure strains were free of flocks, *Bacillus subtilis* forming its characteristic pellicle in our experience only in more concentrated beef extract medium than was here used.

The turbidity was determined by three observers who were unable to distinguish control and thyroxin tubes on the basis of this criterion.

TABLE VI.

(1)	0.075 per cent beef extract + 0.1 per cent desiccated thyroid.	151
(2)	(1) + 0.0001 per cent thyroxin.	149
(3)	(1) + 0.001 " " "	134
(4)	(1) + 0.005 " " "	132
(5)	0.075 per cent beef extract.	104
(6)	(5) + 0.0001 per cent thyroxin.	100
(7)	(5) + 0.001 " " "	103
(8)	(5) + 0.005 " " "	73

The small flagellate present in the mixed cultures was abundant in controls and all three concentrations of thyroxin. *Bacillus subtilis* and *Bacillus fluorescens* were selected to test in pure culture, because it was suspected that if, as Hargitt and Fray (1917) had shown, the former was superior to the latter as a food organism for *Paramecium*, thyroxin might tend to depress the division rate of the former relatively to the latter, and, as both of these bacteria were present in the mixed culture, such a differential action of thyroxin would change the nutritive balance, with a possible effect upon the division rate of *Paramecium*. This suspicion was not supported by the facts.

Further evidence that thyroxin affects *Paramecium* directly, and not indirectly through the food supply is set forth in Table VI which records the total number of divisions of ten lines of *Paramecium* during 10 days, in each of the media listed. The pH of all cultures was adjusted to 7.4 each day, as in the series previously noted.

In both beef extract plus thyroid, and beef extract alone, the depressant action of thyroxin is again apparent in spite of the exceptionally high figure in Table VI under (7).

SUMMARY.

In concentrations of $1:10^4$, $1:10^5$, $1:10^6$, in alkaline solution with a hydrogen ion concentration approximating that characteristic of normal human blood, thyroxin acts like total thyroid in *accelerating certain metabolic processes* in *Paramecium*, notably those connected with the phenomena of excretion.

Unlike total thyroid, thyroxin *depresses the division rate* of *Paramecium*, the degree of depression varying directly with its concentration.

This depression of the division rate is due to the action of the thyroxin directly on *Paramecium*, not to a modification of the food supply.

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THE RELATION OF THE STABILITY OF PROTOPLASMIC FILMS IN NOCTILUCA TO THE DURATION AND INTENSITY OF AN APPLIED ELECTRIC POTENTIAL.

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PLATE 3.

(Accepted for publication, November 26, 1924.)

Present knowledge concerning the changes which occur in a living plant or animal cell when undergoing stimulation is based upon a complex combination of fact and theory. The dominant idea is that some change occurs in the constitution of the protoplasmic film separating the cell from its external medium. One of the features of this change is generally regarded as a reversible increase in permeability. The thickness of the protoplasmic membrane or limiting layer which is involved is not known, and little attention has been given to the important question as to what extent internal phase boundaries of the cell may be involved in such changes of permeability.

In 1908 Nernst[1] first formulated an approximate but clear statement of the relation of duration and intensity of a constant electric current when employed as a stimulus, in the form of the simplified equation $i\sqrt{t} =$ constant, in which i is the intensity of current and t its duration. Attempts at a greater accuracy of expression of this relationship of time and intensity have been made by Lapicque[2] and especially by Hill,[3] and Lucas.[4] However, from more recent results obtained by Jinnaka and Azuma,[5] with a refined experimental

[1] Nernst, W., *Arch. ges. Physiol.*, 1908, cxxii, 275.

[2] Lapicque, L., *J. physiol. et path. gén.*, 1909, xi, 1009.

[3] Hill, A. V., *J. Physiol.*, 1910, xl, 190.

[4] Lucas, K., *J. Physiol.*, 1910, xl, 225.

[5] Jinnaka, S., and Azuma, R., *Proc. Roy. Soc. London, Series B*, 1922–23, xciv, 49, 71.

procedure, it appears that neither Nernst's nor Hill's form of the excitation equation expresses the relations sufficiently accurately for amphibian striated muscle.

In view of these facts it would obviously be of interest to know: (1) what happens in a *visible* protoplasmic film when an electric potential is applied to it; and (2) what is the relation of the duration and intensity of the applied electric potential when this visible protoplasmic film is caused to change in a definite observable manner. The observations which we have made upon *Noctiluca*, and report below, bear upon these questions.

Microscopic Structure of the Cytoplasm of Noctiluca.

The cytoplasm consists of an easily observed emulsion structure in which the large discontinuous phase units, which we shall call phase No. 1, consist of a water solution containing among other things a relatively high concentration of acid, first observed by E. N. Harvey[6] and by Lyon.[7] The average diameter of these vacuoles at the periphery of the cell is about 3 to 6 microns, while the larger, inner vacuoles (clearly shown in the photographs of Plate 3) vary roughly between 10 and 50 microns. The second type of readily distinguishable internal phase consists of faintly pink colored granules, the variable size of which appears to depend in part on the state of nutrition of the cell. Most of these granules occur more centrally about the nucleus while the smaller units of the same kind (1 micron ±) occur scattered in all of the peripheral parts of the continuous phase which forms what we shall call the visible protoplasmic films. The granules in these films move from place to place, always remaining in the films. The whole cell is surrounded by a passive, thin, flexible supporting pellicle secreted by the underlying peripheral protoplasmic film which appears to have a thickness of 1 micron or less and is in continuity with the plasma films of the interior. This peripheral plasma film must, therefore, clearly represent the visible part of the mechanism at the surface of the cell which was shown by E. N. Harvey[6] and Lyon[7] to possess certain peculiar selective

[6] Harvey, E. N., *Carnegie Institution of Washington, Pub. No. 251*, 1917, 235.
[7] Lyon, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 284.

permeability properties, referred to in what follows. It is the behavior of this system of visible peripheral protoplasmic films, under an applied electric potential,which we wish to consider in this paper. The structure described is partly shown in the photographs in Plate 3.

Evidence for Permeability Increase and Coalescence in the Cytoplasmic Emulsion.

If phenol red is added to a suspension of *Noctiluca* cells in sea water no observable change in color of the indicator occurs within at least 20 minutes. However, if the tube is given a strong mechanical shock, a striking color change occurs, indicating loss of acid by some or all of the cells. If this cell suspension is examined under the microscope, some or all of the cells, depending upon the intensity of the shock, are found to have their peripheral cytoplasmic film partly or wholly pulled away from the outermost supporting pellicle. Similarly, as Lyon[7] showed, a brief electric current of sufficient intensity causes the cytoplasm to shrink away from the surrounding pellicle at anode and at cathode, with a simultaneous loss of acid at these points. During these changes the cytoplasm decreases in volume. Careful microscopic examination of this process has shown that this shrinkage occurs as a result of the breakdown of the protoplasmic film or plasma membrane at the periphery of the cytoplasmic emulsion, thus liberating the contents of the peripheral vacuoles of phase No. 1 into the space which forms immediately under the pellicle when shrinkage occurs. The free acid in this space (Figs. 2, 3, and 4, Plate 3) then readily diffuses out through the pellicle, as shown by the indicator. If the electric current is continued this shrinkage progresses until complete or nearly complete coalescence in respect to phase No. 1 has occurred. This picture is quite similar to the precipitation of a coarse viscous emulsion when the process of coalescence occurs at the surface of the emulsion.[8] Now an interesting fact is that the specific gravity of a normal *Noctiluca* cell is less than that of sea water, and therefore it floats at the surface; while the same cell after coalescence of its cytoplasm with respect to phase

[8] Clayton, W., The theory of emulsions and emulsification, Philadelphia, 1923.

No. 1 is denser than sea water and sinks to the bottom of the vessel. From this it is clear that the density of the solution of phase No. 1 is less than that of sea water and yet its effective osmotic concentration must be equal to or greater than that of sea water, providing that the peripheral protoplasmic film separating the solution of phase No. 1 from the sea water does not exert a negative "osmotic force." Facts bearing on this question will be reported in a later paper. The polar coalescence in *Noctiluca* is in certain respects similar to the polar effects of the electric current on *Actinosphærium* and certain other protozoa described long ago by Verworn.[9]

Relation of Duration and Intensity of an Applied Constant Electric Potential to the Process of Anodal Coalescence.

The simplest condition and the only one which will be considered here is that produced when the applied potential is maintained constant throughout its duration (Nernst,[1] Hill,[3] and Lucas[4]). Two experiments performed upon different lots of cells gave practically identical results and are therefore summarized in Table I. Each test was made by noting the duration from the instant when the current was turned on, to the time when one-half of the cells in the field showed beginning of coalescence toward the anode.[10] The duration test for each current intensity was repeated from five to eight times, employing a total of 50 to 90 cells. The values for t given in Table I are therefore averages. Duplicate tests at the same intensity showed a remarkable agreement in the duration of the interval before coales-

[9] Verworn, M., *Arch. ges. Physiol.*, 1895, lxii, 415.

[10] In order to control accurately the density of the electric current flowing through the cells an accurately calibrated wedge-shaped glass chamber was used; the dimensions of which were 14 mm. long, 1.04 mm. deep, 2.2 mm. and 6.25 mm. wide at the two ends respectively. It was provided with a removable glass cover 14 mm. × 10 mm. bearing an accurately calibrated and numbered scale etched on the glass. The current was read to a hundredth of a milliampere on a sensitive ammeter. Products of electrolysis from the electrodes were excluded in a similar manner to that described elsewhere (Lund, E. J., *J. Exp. Zool.*, 1924, xxxix, 357; *Bot. Gaz.*, 1923, lxxvi, 288). Using such an arrangement it is possible to determine quite accurately the threshold potential across any one cell for coalescence. The same section of the wedge was used for all tests reported in this paper.

cence occurred. However, at the extreme lowest and highest intensities of current it was difficult to determine the exact moment when anodal coalescence began in half the cells and, therefore, the first and last values in the table are of somewhat doubtful accuracy. The values for i and t are plotted in the curve in Fig. 1.

The constant k in Nernst's equation is fairly good, in fact better than that which can be obtained from the accurate determinations of Jinnaka and Azuma[5] on frog muscle. It should also be noted that the duration in these experiments is in seconds, while that on muscle by other workers is in 0.001 second. The current density is also apparently much greater than that used by Jinnaka and

TABLE I.

Current intensity. i	Duration. t	$i\sqrt{t}=k$
ma.	*sec.*	
4.20	166 (?)	54.0 (?)
4.90	64	39.2
5.62	38.5	34.8
7.12	30.0	38.9
8.54	20.4	37.5
10.00	16.4	40.4
11.46	13.5	42.0
12.96	10.7	42.3
14.60	9.1	43.9
15.90	9.2	48.1
16.55	7.3	44.9
20.60	5.5± (?)	48.2 (?)

Azuma.[5] The remarkable fact which should be noted is that here we have a process which is represented by a loss of stability of the plasma films, a deemulsification, exhibiting the same relationships to duration and intensity of an electric current as the typical process of stimulation of the striated muscle cell. In fact the agreement in this case with Nernst's physical theory of stimulation appears to be closer than that for the excitation of muscle. The reason for this closer agreement may be due to the fact that in *Noctiluca* the protoplasmic films which Nernst assumed to exist in the muscle cell, are actually realized. Another reason may be that the distances between the visible protoplasmic films in *Noctiluca* are much greater

than those which could reasonably be assumed to exist between intracellular membranes postulated for the muscle cell, and therefore the factor of distance between the membranes, which Hill showed to

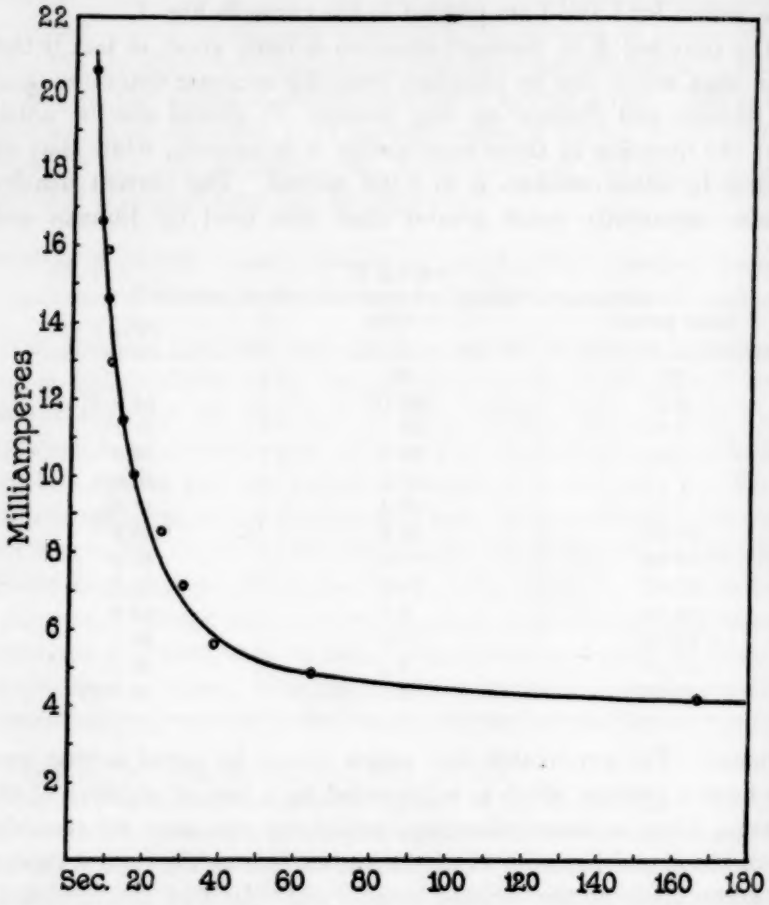


FIG. 1. Curve showing the relation of time and intensity of the constant electric current for coalescence.

be of theoretical importance, is probably less significant in the above experiments on *Noctiluca*.

In typical processes of excitation a living cell usually undergoes a process of recovery. For the present it remains an open question

as to what extent the coalesced cells possess a capacity to recover and therefore whether or not we may consider the changes produced in the plasma films of *Noctiluca* a typical process of excitation. In the writer's opinion the answer to this question at present would therefore merely be a matter of definition. However, the fact which must be emphasized is the striking correspondence between the time-intensity relations in the process of coalescence in *Noctiluca* and in the excitation of the muscle cell.

Another fact which should be noted is that complete coalescence in the plasma films enclosing phase No. 1 does not result in any plainly visible coalescence in the plasma films which surround phase No. 2 (pink granules). Furthermore, E. N. Harvey[6] has shown that *Noctiluca*, after coalescence, continues to respond typically by a flash of light to a mechanical or electrical stimulus. From these facts the significant conclusion would appear to follow that in the process of coalescence described above we have an illustration of *selective coalescence* involving primarily only one visible internal phase (*cf.* below).

Difference in Threshold Intensities at Cathode and Anode.

As far as could be observed the process of coalescence, at the same current intensity, always began earlier at the anode than at the cathode side of the cell, irrespective of the orientation of the cell. This is well shown in Figs. 2, 3, and 4, Plate 3. The visible changes in the plasma films at anode and at cathode do not appear to be different. We may therefore conclude that the threshold differs at anode and cathode because of the difference in the direction of the current through the membrane. Assuming that the current density is the same at the cathode and anode sides of the cell we would be forced to the conclusion that the peripheral plasma membrane is *asymmetric* with respect to those of its properties which are involved in determining the stability of the film when an electric potential is applied to it. This asymmetry is, of course, to be expected since the constitution of the phases (sea water and internal phase No. 1) are demonstrably different with respect to the pH, density, and perhaps other unknown factors such as inherent electric potential.

This difference in threshold stability of the same membrane depending merely upon the direction of the applied current through the membrane at anode and cathode is obviously of interest in connection with the conditions of unidirectional excitation at the nerve synapse.[11]

In order to bring out more clearly the differences between anodal and cathodal coalescence in the same lot of *Noctiluca* cells, data from one series of tests are given in Table II. Tests were made at the current intensities given in the first column of the table. In all the tests the current was passed through the chamber for 1 minute, at the end of which the number of cells showing coalescence only at

TABLE II.

Current intensity. i	Total cells tested.	Cells showing anode effect only.	Cells showing anode and cathode effect.	Cells affected.	Cells affected at both anode and cathode.
ma.				*per cent*	*per cent*
20.04	59	0	59	100	100
18.64	43	0	43	100	100
17.42	56	0	56	100	100
15.90	68	10	58	100	85.2
14.42	85	17	64	95.2	75.2
12.96	92	32	53	92.4	57.6
10.00	94	43	40	88.2	42.5
8.54	93	49	32	87.1	34.4
7.12	83	52	21	87.9	25.3
5.62	64	41	14	85.9	21.8
4.90	64	29	3	50.0	4.6
4.20	70	4	0	5.7	0

the anode, Column 3, and at both anode and cathode, Column 4, were counted. The total number of cells under observation in each test is given in Column 2. From these figures the per cent of the total number of cells showing anodal and cathodal coalescence were obtained, Columns 5 and 6. These percentages are plotted with respect to the corresponding current intensities as graphs in Fig. 2. The curves bring out some interesting facts. (1) The threshold

[11] For a consideration of the relations which exist between direction of an applied P.D. and the direction of an inherent constant P.D. in an epithelial membrane, the reader is referred to the paper by E. J. Lund, *J. Exp. Zool.*, 1925, xli, 155.

current intensity—the time being constant—is higher for cathodal than for anodal coalescence. (2) The total range of variation of threshold intensities for anodal and cathodal coalescence in the cell population is approximately the same, extending from about 4 to

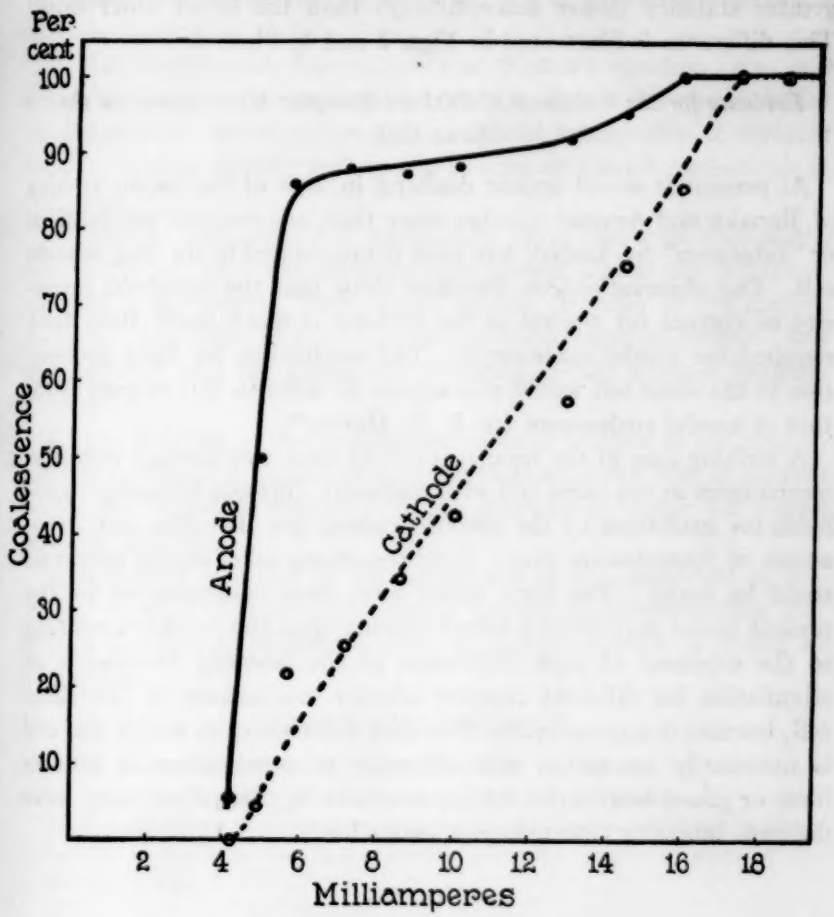


FIG. 2. For explanation see text.

16 milliamperes. This shows very clearly that the stability of the corresponding films in different cells differs greatly. (3) The forms of the curves differ strikingly from one another. The curve for anodal coalescence shows that in a certain small percentage—10 to

15 per cent—of cells, the plasma films are relatively stable compared to those of the remaining 85 to 90 per cent. This difference is in all probability due to a difference in the age of the cell, because it was very clearly evident during the tests that small young cells had a greater stability (lower susceptibility) than the larger older ones. This difference is illustrated in Figs. 2 and 4, Plate 3.

Evidence for the Existence of Distinct Receptor Mechanisms in the Same Cell.

At present it would appear doubtful in view of the recent results of Jinnaka and Azuma[5] whether more than one receptor mechanism or "substance" (*cf.* Lucas[4]) has been demonstrated in the frog muscle cell. Our observations on *Noctiluca* show that the threshold intensity of current for control of the tentacle is much lower than that required for anodal coalescence. The mechanism for light production in the same cell would also appear to differ in this respect from that of anodal coalescence (*cf.* E. N. Harvey[6]).

A striking case of the occurrence of at least two distinct response mechanisms in the same cell with distinctly different intensity thresholds for excitation by the electric current are the cilia and myonemes of *Spirostomum teres*. Doubtless many other similar instances could be found. The facts which have been demonstrated in the present paper may have a direct bearing upon the problems relating to the existence of such differences in the intensity thresholds of stimulation for different receptor-affector mechanisms in the same cell; because it appears quite clear that differentiation within the cell is necessarily associated with diversity in constitution of plasma films or phase boundaries which, as shown in this paper, may have different intensity thresholds for excitation.[12]

SUMMARY.

1. The experiments demonstrate that when a constant electric potential of sufficient intensity is applied to *Noctiluca*, the protoplasmic films which represent a part of the visible continuous phase of the cytoplasm and plasma membrane at the surface of the cell,

[12] Lillie, R. S., Protoplasmic action and nervous action, Chicago, 1923.

become unstable and break down, thus releasing the acid contents of one of the internal discontinuous phases present in the cytoplasm of *Noctiluca*. This process which occurs first at anode then at the cathode side of the cell, appears to be a selective deemulsification or coalescence similar to that at the surface of an emulsion having a viscous continuous phase.

2. The experiments demonstrate that Nernst's equation $i\sqrt{t} = k$ which expresses approximately the relation of duration and intensity of a constant electric current to threshold stimulation of striated muscle, applies equally well to the process of anodal coalescence in *Noctiluca*.

3. Anodal and cathodal coalescence have different thresholds, due to the fact that the semipermeable plasma film at the surface of the cell is asymmetric with respect to the direction of the applied current. Attention is called to the possible relation between this phenomenon and the conditions occurring at the synapse between neurons.

4. The stability of the protoplasmic films in relation to the applied electric potential is greater in young cells than in old cells, or in other words the threshold intensity of the stimulus is higher for young than for old cells.

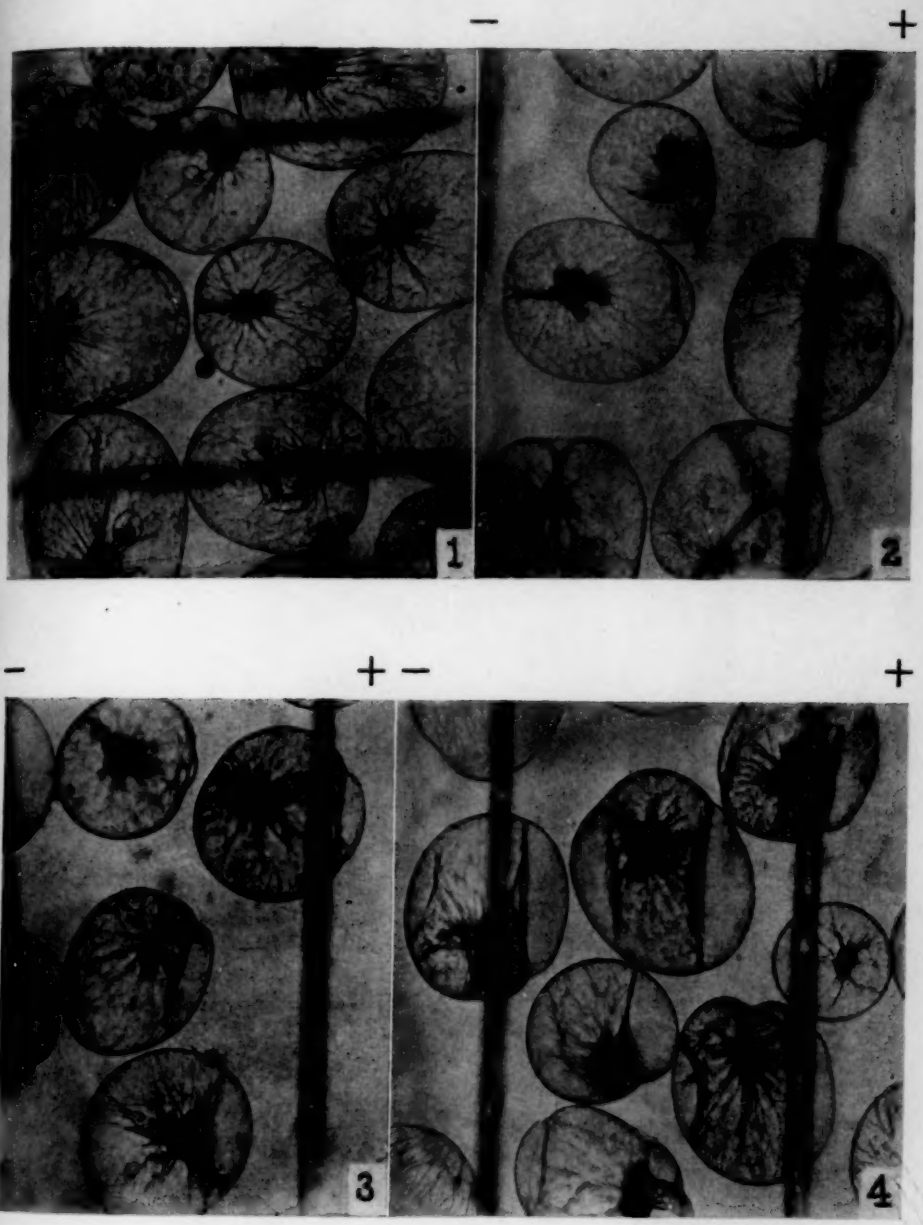
5. Attention is called to the occurrence in the same cell of different receptor-affector mechanisms having a corresponding difference in intensity threshold when an electric current is acting as a stimulus.

EXPLANATION OF PLATE 3.

FIG. 1. Normal unstimulated cells of *Noctiluca*.

FIGS. 2 and 3. Two stages in the process of anodal coalescence.

FIG. 4. Coalescence of the films toward the cathode begins subsequently to the coalescence of the films toward the anode. Note the absence of effect in the small cells in Figs. 2. and 4.



(Lund and Logan: Protoplasmic films in *Noctiluca*.)

FACTORS AFFECTING TRANSMISSION AND RECOVERY IN THE PASSIVE IRON NERVE MODEL.

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The view that the essential factor in the transmission of the state of excitation from region to region in an irritable protoplasmic system (such as nerve) is a form of electrolytic distance action, dependent on the formation of local electrical circuits between the active and the resting regions of the protoplasmic surface,[1] has various implications, some of which can be tested by experiment. It is to be expected, for example, that the speed of transmission will be a direct function of the electrical conductivity of the external medium, since this medium is traversed by the local current between the active and the resting regions. This current, in order to have stimulating effect at a given distance from the active region, must have a certain intensity at that distance, corresponding to the threshold intensity of electrical stimulation under the conditions; evidently this intensity will be decreased if the electrical conductivity of the medium is lowered. According to the local action theory of transmission, the speed (V) of the activation wave is proportional to the product of this critical distance (s) into the rate (r) of the local activation process ($V = sr$); hence any decrease of external conductivity (other conditions remaining equal) must, by decreasing s, decrease correspondingly V. A close proportionality between the conductivity of the medium and the speed of transmission has in fact been observed in the nervous network of the medusa[2] in various dilutions of sea water, and in vertebrate and invertebrate muscle.[3] Since

[1] Lillie, R. S., *Am. J. Physiol.*, 1916, xli, 126.

[2] Mayer, A. G., *Am. J. Physiol.*, 1916–17, xlii, 469; 1917, xliv, 591.

[3] Pond, S. E., *J. Gen. Physiol.*, 1920–21, iii, 807.

lowering the conductivity of the external medium undoubtedly lowers that of the protoplasm as well,[4] such a proportionality is to be expected. This correlation is difficult to explain except on the theory that transmission is dependent on the passage of electric currents through the external medium. It is hardly to be expected that such a correlation will be simple or linear in all cases, since the physical state of the protoplasmic system, *e.g.* the potential difference across the plasma membrane, is altered by changing the electrolyte content of the medium, and other changes affecting irritability may enter and obscure the relation. Its existence in the above cases, however, is clearly favorable to the local action conception of transmission.

The membrane theory (or theories) of stimulation and transmission regards the initial process conditioning both the local stimulation and the spread of the state of excitation to other regions as consisting in a change in the properties of the semipermeable membrane (the diffusion-proof interfacial film) bounding the surface of the irritable protoplasmic element (axone, fibril, etc.). This film undergoes locally, during excitation, a temporary breakdown or increase of permeability.[5] According to the local action theory, this change, by altering the potential across the film (membrane or other potential), gives rise to a local current between the altered region and the unaltered region adjoining, which is then secondarily stimulated. In this secondary stimulation the film is altered in the same manner as at the original site of stimulation; hence the same effect is repeated at each activated region and the state of excitation spreads. What is essential to note is that the self-propagating character of the excitation process depends on the alteration of film structure involved in the excitation process itself; the structural change in the film produces directly an electromotor variation, and hence a local current; the latter by means of its electrochemical effects in adjoining regions causes the structural change in the film, and hence the local excitation. The electrical sensitivity of the protoplasmic system

[4] Brooks, S. C., *J. Gen. Physiol.*, 1922–23, v, 365.

[5] The evidence that stimulation is associated with changes in the permeability and other properties of the protoplasmic films is reviewed in my recent volume, Protoplasmic action and nervous action, Chicago, 1923, *cf.* Chapter 14.

thus depends on the presence of films which undergo chemical and structural change under the influence of the local bioelectric currents. Such chemical effects depend ultimately, as in the case of reactions at electrodes in general, on changes of polarization and transfer of electrons between the phases.

In the inorganic system, passive iron in dilute nitric acid, which I have recently used as a model of the transmitting protoplasmic system,[6] transmission is known to depend on the electrochemical effect of the local current between the active and the passive regions of the metal. The latter region, being cathodal, is activated by electrolytic reduction of the passivating film of oxide; reduction involves breakdown of the film; and since this effect is repeated wherever active and passive regions adjoin each other, the activation reaction spreads rapidly over the whole surface, provided the film is uniform in its properties and readily reducible at every point. Any region locally activated, mechanically or otherwise, thus becomes the point of departure of an activation wave. The rate of travel of this wave is a direct function of the electrical conductivity of the local active-passive circuit; theoretically, it is proportional to the product sr, s being the maximal distance beyond the active-passive boundary at which the local current is sufficiently intense to remove or disrupt the film, and r being the rate of removal at that region. The rate of transmission is thus a direct index of the rate of removal or breakdown of the film.

In a completely transmissive ("recovered") wire immersed in acid of uniform composition and temperature such an activation wave travels for an indefinite distance at almost uniform velocity. In acid above a certain strength (*ca.* 55 volumes per cent HNO_3 of sp.gr. 1.42) the wire spontaneously reverts to the passive state immediately after the passage of the activation wave; *i.e.*, the film is immediately reformed, the exact time required depending on the temperature and the concentration of acid.[6] Some further time, however, is needed for the return of the newly formed film to its original condition; during this interval, ("recovery interval") transmission is incomplete (decremental) or slower than before.

[6] Lillie, R. S., *Science*, 1918, xlviii, 51; 1919, l, 259, 416; *J. Gen. Physiol.*, 1920–21, iii, 107, 129.

The passive iron wire in nitric acid is an example of a heterogeneous system in which the rate and character of the chemical reactions are controlled by variations in the composition and properties of films formed or deposited at the surface between the phases. Mercury in H_2O_2 is another such system. Reaction is initiated when the film is locally removed or disrupted, and comes to rest when the film is restored. When the formation and dissolution of the film are rhythmical, the reaction is also rhythmical. It appears highly probable that a similar condition exists in living protoplasm, in the sense that variations of film structure control the chemical interactions between the protoplasmic phases, or the reactions occurring at the surfaces of the protoplasmic structures. Reactions of this type almost certainly underlie the response to stimulation. Many metabolic reactions are known to be dependent on the intact structure of the protoplasmic system;[7] there is also much evidence of variation in the structure and permeability of the protoplasmic films during stimulation or other activity.[5] The resemblances between the phenomena of activation and transmission in protoplasmic systems (such as nerve) and in the passive iron system are an expression of this general similarity in the physical conditions controlling the chemical reactions in the two systems. Such resemblances are independent of special features of chemical composition. It is well recognized that the study of polyphasic systems of a simple class, such as emulsions and similar colloidal systems, is of fundamental importance in relation to many general problems of physiology. The same is true of systems of the class to which passive iron belongs. The passive iron system is a polyphasic (specifically a triphasic) system, peculiar in the respect that its chemical and physical activity is largely determined by the formation and removal of interfacial films, and by variations in the properties of these films. Systems of this class are of special interest to the physiologist; they are *models*, in which purely chemical complexities are reduced to a minimum, while the structural conditions controlling reaction velocities and the transmission of chemical influence to a

[7] For a review of the evidence *cf.* Meyerhof, O., Chemical dynamics of life phenomena, Monographs on experimental biology, Philadelphia and London, 1924.

distance are apparently of the same general kind as those existing in living protoplasm.

The chief results of the present study may be conveniently considered under the following heads.

1. Factors affecting speed of transmission: (*a*) conductivity of external medium; (*b*) temperature; (*c*) length of interval since previous activation.
2. Influence of temperature on recovery of transmissivity.
3. Structural conditions affecting speed of transmission.

Method.

The speed of transmission in a passive iron wire immersed in a large volume of nitric acid is too rapid for accurate measurement by ordinary means.[8] When, however, the sectional area of acid is small, as in a wire suspended in air with only a thin layer of acid adhering, the speed is reduced to a few centimeters per second and the progress of the activation wave can then readily be followed by the eye. The method employed in the present study has been to measure the speed of transmission in wires inclosed in glass tubes of known diameter. The sectional area of electrolyte and hence the conductivity are thus known; transmission is slower the smaller the diameter of the tube; and if wires of sufficient length and tubes of appropriate diameter are used, the speeds are easily measured with a stop-watch. For example, in a tube of 3 mm. caliber the average speed of transmission (in the wires used in the present study) is about 26 ± 2 cm. per second in completely recovered wires at 20°. Since the actual variation in the speed of transmission in the wires is greater than the variation of stop-watch measurements (assumed to be accurate within 0.2 second), this simple method gives all the exactitude necessary. Other possible methods, such as measuring the rate of travel of the electromotor variation by the string galvanometer, while more accurate in single determinations, are too inconvenient and time-consuming for use in an investigation such as the present, which requires the averages of a large number of

[8] Estimated as some meters per second in a completely recovered wire in a large volume of 70 per cent HNO_3 at 20°.

determinations. The speed of transmission in single wires under constant conditions has been found subject to considerable irregular variation, often ranging 10 per cent or more from the mean value (see Table II). This variability is to be referred to chance irregularities in the structure of the metal, such as inequalities in the distribution of the carbon in the steel (which is essentially polyphasic or colloidal in structure), or inequalities due to strain. It is evident that a new metallic surface is formed in each activation, since a thin layer of iron is then dissolved. Hence, each observation is made under conditions somewhat different from those of the preceding observation and not subject to control. In order, therefore, to secure consistency in the measurements, it is necessary to make numerous observations and take the average of the results (with consideration of probable errors). It is also important to use wires which are as nearly as possible in the same physical condition (see below, page 481).

In studying the relation of speed of transmission to external conductivity the most satisfactory method is to vary the diameter of the glass tubes containing the wires, while keeping the strength of acid constant. The alternative and perhaps more obvious method, that of measuring the speed in tubes of a constant size containing acid of varying concentrations, is inapplicable, for the reason that the character of the local reaction and the rate of repassivation and recovery both vary widely with the strength of acid. The recovery of complete transmissivity becomes more gradual as the strength of acid increases, being about twice as rapid in 70 per cent as in 80 per cent HNO_3;[9] while the local chemical effects of activation (effervescence, formation of dark oxide) are more pronounced in weaker acid.[10] The breakdown of the film (the basis of activation) and its reformation (the basis of repassivation and recovery) are chemically distinct processes, differently affected by temperature and concentration of acid. Evidently in experiments of the present kind it is important to maintain as nearly constant as possible the purely chemical features of the local reactions. A brief local reaction and a rapid recovery are desirable,

[9] Lillie, R. S., *J. Gen. Physiol.*, 1920–21, iii, 107; *cf.* curve on p. 125.
[10] Lillie,[9] pp. 113, 114.

and these conditions are satisfactorily met in acid of 70 per cent concentration. In this solution, the local reaction lasts for less than a second, the darkening of the metallic surface is sufficient to mark plainly the progress of the activation wave, and the recovery of complete transmissivity is relatively rapid, occupying about 5 minutes at 20°. Accordingly this concentration (70 volumes per cent of C. P. HNO_3, sp.gr. 1.42) has been used uniformly throughout the experiments described in this paper.[11]

The following measurements have all been made on passive wires inclosed in four tubes of 38 cm. length and of the respective inner diameters, 2.4, 3.0, 3.5, and 3.9 mm. In these tubes the speeds of transmission are of an order suitable for measurement with a stop-watch. The four tubes are placed side by side, a few centimeters apart, in a long, narrow, flat bottomed, thick walled porcelain trough (*ca.* 100 × 15 cm.) containing a shallow layer (*ca.* 1.5 cm. deep) of 70 per cent HNO_3. The temperature of the acid is controlled by surrounding the porcelain trough with water, or a mixture of ice and water, contained inside a larger wooden copper-lined trough (120 × 25 × 15 cm. deep). By adding water or ice to the outer trough as required the temperature of the acid can readily be kept constant within 0.5° for any desired period. Measurements of speeds of transmission were made at the four temperatures, 5°, 10°, 15°, and 20°.

It is well known that a passive iron wire in nitric acid shows only a partial or limited (decremental) transmission for a certain interval of time (usually several minutes) immediately following the passage of an activation wave.[6] The exact duration of this interval depends on the concentration of acid and the temperature, being longer for stronger acid and lower temperatures. In 70 per cent HNO_3, in wires not inclosed in tubes, an interval of about 6 minutes is required (at 20°) for the recovery of complete or non-decremental transmission. It had also been observed that for a certain time after the wire has become completely transmissive the speed of

[11] The presence of ferric salt (from dissolved wire) seems to influence the rate of the reaction, and in my experience "used" acid (colored somewhat deeply with ferric salt) has given more regular results than freshly mixed acid. Hence I have used such acid throughout.

transmission is slower than previously to activation. During this period, lasting about 15 minutes at 20°, the speed increases progressively to a maximum. In order to determine the relation between the speed of transmission and the conductivity (or the temperature) it is, therefore, necessary to make the observations in the different tubes at corresponding times; *i.e.*, (at a given temperature) at equal intervals after a previous activation. At such times the state of the passivating film in the four wires is presumably the same, and the wires are in a comparable physical state.

In each experiment the procedure has been as follows: The four passive wires, each *ca.* 45 cm. long, are transferred by forceps[12] from the passivating trough[13] to the experimental trough. They are then activated in regular order by touching with a zinc rod, the intervals between successive activations being exactly 1 minute.

FIG. 1. Position of wire and enclosing tube in trough of acid.

Each wire is then inserted into its tube in such a manner that its left end is on a level with the left end of the tube while its right end projects into the acid outside. In order to insulate the wire at one end from the external acid, the left end of each tube is supported slightly above the general level of the acid[14] (by resting across a piece of thick walled glass tubing); the other end (from which the wire projects) is immersed in the acid (Fig. 1). After the lapse of the definite interval allowed for recovery the wires are again activated in the same order, by touching the projecting ends with the

[12] Steel forceps are used, the ends of which are bound with platinum foil to prevent activation. During use these forceps are kept passive by immersing the ends in a beaker containing strong HNO_3.

[13] Between experiments the wires are kept passive in a separate trough containing 70 per cent HNO_3.

[14] It is necessary to have one end of the wire insulated, *i.e.* out of contact with the general body of acid, since otherwise when one end is activated the activating influence is transmitted instantaneously by distance action through the acid and an activation wave is started at both ends of the tube (see below, p. 502).

zinc rod, allowing 1 minute between successive activations as before. All wires are thus activated at the same interval after the previous activation. The passage of the wave of activation along the tube (from right to left) is accompanied by momentary effervescence and darkening of the metallic surface. The boundary of the activated area is plainly visible as it travels, and its exact time of passage from end to end of the tube is measured with the stopwatch. Immediately after activation, each wire is withdrawn from its tube and allowed to lie in the acid. The acid inside the tube is then changed,[15] and the wire is replaced in its tube in time for the next determination. With practice the acid can easily be changed and the wire replaced during the minute intervening between successive activations. When four wires are used the least possible interval between successive activations of the same wire is thus 4 minutes. No purpose, however, would be served by making this interval shorter, since 4 minutes (approximately) is the least time in which a wire (inclosed in a tube at 20°) can recover complete transmissivity. The shortest interval allowed for recovery in the experiments described below was 5 minutes (at 20°).

The kind of wire used was the same as in my previous study on recovery of transmissivity.[16] This wire is *ca.* 1.2 mm. in diameter. Experience has shown that more uniform results are obtained in different experiments if wires are used in which the outer layer of metal has been dissolved away by acid. Accordingly the data of Tables II, III, and IV are all from experiments with used wires—*i.e.* those which had been reduced to two-thirds or less of their original diameter (*i.e.*, from 1.2 to 0.8 mm. or less). Freshly cut wires transmit more slowly than used wires. This difference is illustrated in Table I, which gives, under Series A, the seconds required for transmission along each of the four tubes in four successive activations with freshly cut wires, allowing 6 minutes for recovery in each case, and under Series B, similar measurements

[15] By lifting one end of the tube, allowing the acid to flow out, and then returning the tube to a level to allow fresh acid to flow in (avoiding bubbles); this procedure is repeated two or three times if necessary.

[16] No. 20 piano wire (music steel wire) from the Spencer Company of Worcester Massachusetts.

made on the same wires after they had been reduced to two-thirds or less of their original diameter. Each horizontal line gives four successive determinations with the same wire in the same tube, after the same period of recovery.

It is not quite clear why the freshly cut wire should transmit so much more slowly than the wires with surface layer removed.[17]

TABLE I.

Wires in 70 per cent acid at 20°. Four successive activations of each wire; interval since previous activation, 6 minutes in each case. Length of tubes, 38 cm.

Series A. Freshly cut wires (1.2 mm. diameter); activations of wire in Tube A at (2.28), 2.34, 2.40, 2.46, and 2.52 p.m., Jan. 29, 1924.

Diameter of tubes.	Sec. for transmission along tubes.				Average speed of transmission (cm./sec.).
	(1)	(2)	(3)	(4)	
mm.					
A. 2.4	4.2	4.4	4.2	4.4	8.8
B. 3.0	3.2	2.8	3.0	3.2	12.7
C. 3.5	2.0	2.4	2.6	2.4	15.8
D. 3.9	1.8	1.6	1.8	1.8	21.1
Average for the four tubes					14.6

Series B. The same wires after reduction to a diameter of *ca.* 0.75 mm.; activations of wire in Tube A at (3.15), 3.21, 3.27, 3.33, and 3.39 p.m., Feb. 11, 1924.

A. 2.4	1.8	2.2	2.8	1.8	18.1
B. 3.0	1.6	2.2	1.6	2.2	20.0
C. 3.5	1.4	1.6	1.8	2.0	22.3
D. 3.9	1.2	1.4	1.4	1.2	29.2
Average for the four tubes					22.4

The bracketed time is the time at which the wire in Tube A was activated before the first recorded observation was made.

Possibly local circuits dependent on the chance distribution of the carbon in the steel may play a part in delaying transmission; contact of carbon or platinum with a passive wire is known to retard or block the activation wave.[18] The metal may be more homogeneous

[17] The increase in the sectional area of acid is entirely insufficient to account for this difference.

[18] *Cf.* Lillie, R. S., *J. Gen. Physiol.*, 1920–21, iii, 137.

near the axis; if this is true, a partly dissolved wire would form fewer irregular circuits. In any case my experience has been that the behavior of the wires becomes more regular as the outer layer is removed.[19]

Accordingly I have used as a control throughout the present study a preliminary test in which the time of transmission has been determined at 20° in all four tubes, after a recovery interval of 6 minutes. Only those wires have been used in which the average speed of transmission under these conditions was 20±2 to 3 cm. per second. Within this range of variation the behavior of such wires is constant and predictable under definite conditions of temperature and external conductivity; and if the average of a sufficient number of observations is taken, the influence of chance irregularities becomes unimportant. Table I illustrates the kind of irregularities observed in these determinations; these represent real variations of speed and not errors of measurement; that this is the case is seen most clearly at lower temperatures and in the narrower tubes, where the systematic error (*ca.* 0.2 second) of stop-watch measurement is relatively slight. The activation wave rarely moves with perfect uniformity along the tube, but shows slight accelerations and retardations at intervals. Apparently, these fluctuations are the expression of variations in the structure of the metallic surface, as already indicated.

EXPERIMENTAL.

Using wires selected in this manner, and the same series of four tubes, I have made a large number of measurements of speeds of transmission at the four temperatures 5°, 10°, 15°, and 20°. At each temperature the interval since the previous activation was varied through nearly the whole range between the minimal time required for the recovery of non-decremental transmission and the time required for complete return to the original speed. Table II gives the results of four typical series of observations at the four temperatures.

These observations are typical of the behavior of the wires at the

[19] *Cf.* Lillie, R. S., *Science*, 1919, l, 417.

TABLE II.

Approximate speeds of transmission, in cm. per sec., as measured by the stop-watch under the conditions already described. Data are given from four separate series of experiments, carried out respectively at 5°, 10°, 15°, and 20°. Each horizontal line gives observations made on the same wire, in the same tube, and at the same temperature, but at varying intervals after a previous activation (recovery intervals). The figures represent typical single observations and not averages. (Averages of all experiments are given in Table III.)

Series I. Temperature 5° (series of Mar. 10, 1924).

Diameters of tubes.	Speeds of transmission (cm./sec.) after recovery intervals.							
	12 min.	16 min.	20 min.	30 min.	45 min.	1 hr.	1 hr. 30 min.	Some hrs. (complete recovery).
mm.								
A. 2.4	6.6	8.0	8.3	10.6	11.9	11.2	12.7	14.6
B. 3.0	10.0	11.2	11.2	12.7	14.6	14.6	17.3	17.3
C. 3.4	11.2	12.7	12.7	15.8	17.3	17.3	19.0	21.1
D. 3.9	11.2	11.2	13.8	15.8	19.0	19.0	23.7	23.7

Series II. Temperature 10° (series of Feb. 25 to 27, 1924).

	8 min.	12 min.	16 min.	20 min.	30 min.	45 min.	1 hr.	Some hrs.
A. 2.4	9.0	11.2	11.9	12.7	13.8	15.8	15.8	14.6
B. 3.0	11.9	15.8	15.8	15.8	15.8	19.0	21.1	21.1
C. 3.4	13.8	11.8	17.3	17.3	21.1	23.7	21.1	23.7
D. 3.9	13.8	17.3	21.1	21.1	21.1	23.7	27.1	27.1

Series III. Temperature 15° (series Mar. 31 to Apr. 4, 1924).

	6 min.	8 min.	12 min.	16 min.	20 min.	30 min.	45 min.	1 hr.	Some hrs.
A. 2.4	6.3	9.5	13.8	14.6	15.8	17.3	19.0	19.0	19.0
B. 3.0	9.0	13.8	15.8	19.0	19.0	21.1	23.7	23.7	23.7
C. 3.4	13.8	17.3	21.1	23.7	27.1	27.1	27.1	27.1	27.1
D. 3.9	14.6	17.3	23.7	23.7	27.1	27.1	31.7	31.7	31.7

Series IV. Temperature 20° (series of Feb. 6, 1924).

	5 min.	6 min.	8 min.	10 min.	12 min.	14 min.	16 min.	Fully recovered.
A. 2.4	9.0	11.2	14.6	17.3	19.0	21.1	23.7	23.7
B. 3.0	13.8	14.6	17.3	23.7	23.7	23.7	23.7	27.1
C. 3.4	14.6	15.8	21.1	23.7	27.1	31.7	31.7	31.7
D. 3.9	19.0	21.1	23.7	27.1	31.7	38.0(?)	31.7	31.7

TABLE III.

Average speeds of transmission in cm. per sec. at the four temperatures, 5°, 10°, 15°, 20°, after varying recovery intervals. The data represent averages of all observations made under comparable conditions, as above defined. In each experiment used in computing these averages observations were made in all four tubes under identical conditions of recovery time and temperature; *i.e.*, under conditions like those indicated in each series of Table II. The bracketed numbers in the first column give the number of separate experiments with each recovery interval; the number of separate observations averaged in the last column is accordingly four times this number. The total number of experiments is 354.

Recovery interval.	Average speed in each tube (cm./sec.).				Average speed in all four tubes (cm./sec.).
	A (2.4 mm.)	B (3.0 mm.)	C (3.5 mm.)	D (3.9 mm.)	
Series I. Temperature 20°.					
5 min. (10)	10.6	13.8	17.3	20.0	14.6
6 " (15)	14.6	18.1	21.1	27.1	19.0
7 " (8)	15.2	15.8	20.0	25.3	18.1
8 " (10)	15.8	19.0	22.3	25.3	20.0
9 " (6)	15.8	21.1	25.3	29.2	22.3
10 " (9)	17.3	21.1	25.3	29.2	22.3
12 " (12)	20.0	22.3	27.1	31.7	25.3
14 " (10)	22.3	25.3	29.2	31.7	27.1—
15 " (4)	22.3	23.7	31.7	34.5	27.1
16 " (6)	23.7	23.7	31.7	34.5	27.1
1–2 hrs. (3)	23.7	27.1	31.7	34.5	27.1
Average.....	18.3	21.0	25.7	29.4	
Series II. Temperature 15°.					
6 min. (11)	7.3	9.3	12.3	12.7	10.0
7 " (4)	9.3	11.2	13.1	14.1	11.5
8 " (17)	9.7	12.3	14.1	16.9	12.7
9 " (5)	10.0	13.1	14.6	16.9	13.1
10 " (11)	10.9	14.1	16.9	19.0	14.6
12 " (12)	11.9	14.6	17.3	20.0	15.2
14 " (7)	14.1	16.9	22.3	23.7	18.1
16 " (6)	14.1	18.1	22.3	25.3	19.0
20 " (8)	15.8	19.0	23.7	27.1	20.0
25 " (7)	16.9	20.0	25.3	27.1	21.1
30 " (7)	16.9	22.3	27.1	27.1	22.3
45 " (7)	20.0	23.7	29.2	31.7	25.3
60 " (3)	20.0	25.3	29.2	31.7	25.3
75–90 " (3)	20.0	23.7	29.2	31.7	25.3
Average.....	14.1	17.4	21.2	23.2	

TABLE III—*Concluded.*

Recovery interval.	Average speed in each tube (cm./sec.).				Average speed in all four tubes (cm./sec.).
	A (2.4 mm.)	B (3.0 mm.)	C (3.5 mm.)	D (3.9 mm.)	
Series III. Temperature 10°.					
8 min. (27)	8.1	10.9	11.2	14.6	10.0
10 " (13)	9.3	11.2	12.3	15.2	11.5
12 " (13)	10.3	13.1	14.6	16.9	13.1
14 " (10)	10.9	13.8	16.9	19.0	14.6
16 " (8)	12.3	14.1	18.1	21.1	15.8
20 " (7)	12.3	14.6	18.1	21.1	15.8
25 " (3)	14.1	14.6	18.1	23.7	17.3−
30 " (9)	13.1	16.9	20.0	23.7	17.3+
45 " (8)	14.1	18.1	22.3	25.3	19.0
60 " (8)	15.2	19.0	22.3	27.1	20.0
90 " (6)	17.3	20.0	23.7	27.1	21.1
Some hrs. (6)	18.1	22.3	25.3	27.1	22.3
Average.....	13.0	15.7	18.6	21.8	
Series IV. Temperature 5°.					
12 min. (5)	6.3	9.0	10.9	11.2	8.8
16 " (5)	7.8	10.3	12.3	12.3	10.3
20 " (5)	8.0	10.9	12.3	13.1	10.9
30 " (5)	10.3	11.2	14.1	15.2	12.3
45 " (5)	11.2	13.8	17.3	18.1	14.6
60 " (5)	11.9	14.6	17.3	19.0	15.2
90 " (5)	13.1	17.3	19.0	21.1	17.3
Several hrs. (6)	14.1	17.3	21.1	23.7	19.0
Average.....	10.3	13.0	15.5	16.7	

four temperatures. The chief features to be noted as of general interest are as follows:

1. The speed of transmission increases regularly with the diameter of the tube. This increase, however, is more gradual than the increase in the sectional area of electrolyte; *i.e.*, than the external conductivity. As will be shown below, it is nearly proportional to the square root of the conductivity.

2. At each temperature the speed increases progressively with increase in the interval since activation (recovery interval), at first rapidly, then more slowly, until a maximum is reached.

3. The time required to reach this maximum decreases rapidly with rise of temperature; at 5° it is 90 minutes or more, at 10° *ca.* 1 hour, at 15° between 30 and 45 minutes, at 20° *ca.* 16 minutes. The temperature coefficient of the recovery process is thus high.

4. No relation can be seen between the caliber of the tubes and the rate of recovery; *i.e.*, recovery is independent of external conductivity.

5. The influence of temperature on the speed of transmission is relatively slight, (Q_{10} being of the order 1.3 to 1.5). There is thus a

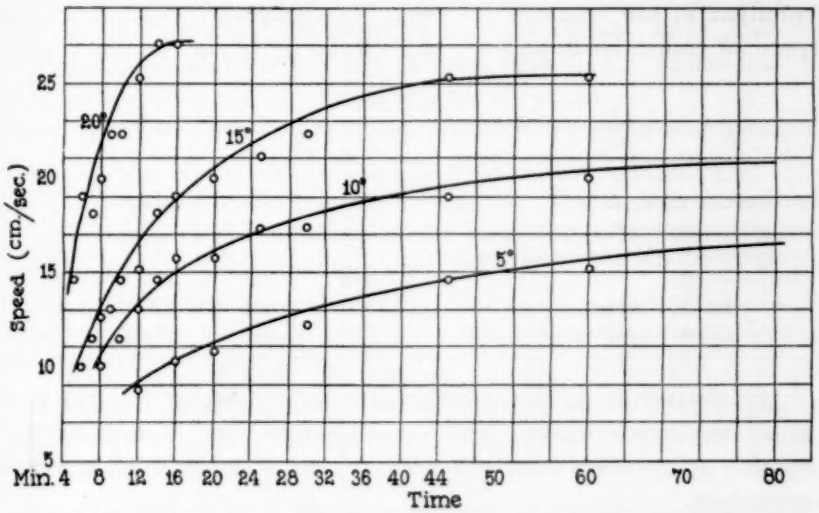

FIG. 2. Curves showing the relation between speed of transmission and recovery interval (time elapsed since previous activation) at the four temperatures, 5°, 10°, 15°, and 20°. The points are the averages in the last column of Table III.

low temperature coefficient for transmission and a high one for recovery, a condition similar to that found in nerve and muscle.

Table III gives the averages of the speeds of transmission observed in each of the four tubes in all experiments made under comparable conditions as above defined. The number of experiments performed with each recovery interval is given in brackets in the first column. In the last column are given the averages of the speeds in all four tubes, after the same recovery interval. These averages give the most accurate index of the general relations between speeds of

transmission and recovery times at the different temperatures. The data are represented graphically in Fig. 2.

Relation between Speed of Transmission and Conductivity of External Medium.

The averages at the foot of each column in the four series of Table III show that the ratios between the speeds of transmission in the four tubes are practically unaffected by variation of temperature within the range 5–20°. They also show no change with variation in the recovery interval. The general relation between speed of transmission and external conductivity, *i.e.* sectional area

TABLE IV.

	A	B	C	D
Diameters of tubes, *mm*	2.4	3.0	3.5	3.9
Sectional areas of acid, *sq. mm*	4.0	6.6	9.1	11.5
Average speeds (354 experiments), *cm./sec.*	11.9	14.6	17.3	20.0
Relative speeds	100	123	146	168
" sectional areas	100	163	227	287
" square roots of sectional areas	100	128	151	169

of the electrolyte, is thus best ascertained by taking the averages of all the measurements made in each tube, and comparing the relative values thus obtained with the relative sectional areas of electrolyte.[20]

With a tube of radius t inclosing a wire of radius w, the sectional area of acid is evidently $\pi(t^2-w^2)$. In Table IV are given the sectional areas of the acid in the four tubes, together with the observed speeds of transmission (the averages of 354 observations made under similar conditions in each tube). The relative speeds and conductivities are also given, expressed as percentages of the

[20] Since equal numbers of measurements are made in the four tubes, and the conditions (of temperature, recovery interval, and composition of acid) are always the same in the four observations of each experiment, this use of the averages is legitimate. The result gives the average ratios of the speeds in the four tubes under a wide range of conditions. Evidently the ratios of the four conductivities are the same throughout the whole series.

values in Tube A (neglecting decimals); also the relative square roots of the conductivities.

Since transmission is the direct result of the electrolytic breakdown or removal of the passivating film by the local active-passive current, and since according to Ohm's law the distance from the active-passive boundary at which the current has a definite intensity—*e.g.* the critical minimum required to reduce the film—should vary directly with the conductivity of the circuit, it might be expected that the rate of transmission would be directly proportional to this conductivity; *i.e.*, to the sectional area of the electrolyte. In fact, however, the rate of transmission increases more slowly than the conductivity, and very nearly in proportion to the square root of the latter. What is the reason for this discrepancy?

Apparently there are two conditions that make the speed of transmission in a narrow tube greater than would be expected from a consideration of the conductivity alone. One is the relation between the electrical resistance of the local couple and the steepness of the potential gradient between the two electrode areas of the transmitting wire (*i.e.*, the active and passive areas immediately adjacent to the boundary). When the resistance is low this gradient is less steep than when the resistance is high, somewhat as in the analogous case of a battery which is cross-circuited, as compared with one discharging through a high resistance. Hence the potential between a point in the passive area at a given distance from the boundary and a point similarly situated in the active area will be greater in a narrow than in a wide tube. The region in the passive area where the current has the critical intensity required to reduce the film will thus extend farther from the boundary in a narrow tube than the conditions of resistance, considered alone, would indicate. There is, however, no evidence that this factor plays any important part in tubes of the above dimensions.[21]

The other condition is that the effective electrode area on the

[21] When the layer of acid is very thin, as in the case of a wire removed from the acid and held suspended vertically in the air, this factor becomes important, and the rate of transmission in such a wire is greater than one would expect from the thickness of the acid layer. I have not studied systematically the rates of transmission in tubes narrower than 2.4 mm.

passive side of the boundary, *i.e.* the area in which the film is in process of being removed, is not the same in the four tubes, but becomes progressively smaller as the diameter of the tube increases, because of the direct action of the current itself in removing the film. The rapid removal of the film limits the effectiveness of the current in removing further film in the passive area immediately beyond the boundary, since the reacting portion of the surface—that in which the actual reduction of the oxide layer is proceeding—is thus continually being diminished. The rate of removal of the film is what determines the rate of transmission; hence any such condition, by imposing limits on the rate of removal, limits correspondingly the rate of transmission.

This second condition appears to be the chief factor in the above discrepancy, as the following simple calculation shows.[22]

The rate at which the activation wave travels is a direct measure of the rate at which the film is being removed at the cathodal area adjacent to the active-passive boundary. This rate depends on the intensity of the current at the area in question. Now the intensity of this current, I, is determined, as in a battery, by the general conditions

$$I = PCA$$

in which P is the potential between the electrodes, C, the conductivity of the local circuit, and A, the electrode area. But A is continually being diminished by the action of the current, and the more rapidly the more intense the current. If we assume that this reduction of area is directly proportional to the intensity of current, so that the effective electrode area is inversely proportional to this intensity, the following relations hold:

$$A = \frac{K}{I}$$

(in which K is a proportionality constant)

$$I = \frac{KPC}{I}$$

[22] I am indebted to Dr. Richard Rüdy of the Nela Research Laboratories for calling my attention to this relation.

$$I = \sqrt{KPC}$$

Since K and P are constants, the expression may be written $I = k\sqrt{C}$; *i.e.*, the intensity of the current in the area undergoing alteration, and hence the rate of removal, vary directly with the square root of the conductivity.

Since transmission depends on the removal of the film, its speed, S, follows the same law; *i.e.*, $S = k\sqrt{C}$. This approximation is not perfect, but seems sufficiently close to indicate that the divergence from direct proportionality between speed and conductivity is a result of the tendency of the effective electrode area on the passive side of the boundary to become proportionately smaller as the intensity of the local current increases.

Relation between Speed of Transmission and Temperature.

In order to determine this relation, it is necessary to compare the speeds of transmission (at the different temperatures) in wires which are in corresponding stages of recovery. This is best done by allowing the wires to recover completely, *i.e.* to attain the maximal speeds of transmission, before making the measurements. The wires may be left undisturbed in acid at room temperature (20°) for 16 minutes or more and then be transferred to acid at the temperature desired, or they may be left for a sufficient period in acid which is already at the temperature of the experiment.

The average speeds in completely recovered wires at the four temperatures, according to the experiments summarized in Table III, are as follows:

Temperature. (°C.)	Speed. (cm./sec.)
5	19.0
10	22.3
15	25.3
20	27.1

Taking the different pairs of observations and applying the formula $Q_{10} = \left(\frac{V_{t_1}}{V_{t_2}}\right)^{\frac{10}{t_1 - t_2}}$ the values of Q_{10} are found to be between 1.3 and 1.5 (except for the interval 15–20° where it is *ca.* 1.2).

In a later separate series of experiments in which six determinations were made with each tube at the above temperatures (*i.e.* 24 determinations in all at each temperature), using the same set of four uniformly acting wires throughout, the average speeds observed were as follows:

Temperature. (°C.)	Speed. (cm./sec.)
5	18.1
10	22.3
15	27.1
20	34.5

In this series the Q_{10} values lie between 1.4 and 1.6.

From these results it is evident that the temperature coefficient of transmission is low, of the order $Q_{10} = 1.4$ to 1.5. The significance of this fact seems clear. Transmission depends on an electrochemical reaction in a local circuit; it is therefore affected by temperature in the same manner as the electrochemical reactions in a battery or other galvanic element. The speed of transmission depends on the local intensity of current; this intensity is determined by Ohm's law $\left(I = \frac{P}{R}\right)$. Both potential and resistance are relatively slightly affected by change of temperature. The former varies with the absolute temperature $\left(P = \frac{RT}{nF} ln \frac{c_2}{c_1}\right)$; while the Q_{10} of conductivity of the electrolyte is similar to that of diffusion, of the order 1.2 to 1.3. Hence the temperature coefficient of the active-passive couple is low, like that of a battery, and this coefficient determines that of transmission. It is possible, however, that the presence of superposed or accessory reactions other than electrochemical may make the total temperature coefficient of transmission somewhat higher than that of a purely electrochemical reaction.

It is noteworthy that the Q_{10} of transmission in nerve and muscle is of a similar order, $Q_{10} = 1.6$ to 1.8;[23] and this fact lends further support to the view that in the protoplasmic system transmission

[23] For a summary of the data *cf.* Kanitz, A., Temperatur und Lebensvorgänge, Berlin, 1915.

is also essentially the result of a local electrochemical reaction, in which the surfaces of the protoplasmic phases play the part of electrodes. In this case, too, there are probably superposed or side reactions which affect the temperature coefficient of the total process and make it somewhat higher than it would be if the electrochemical reactions were the only ones concerned.[24] In any event it is significant that the temperature coefficients of transmission and of recovery differ in the protoplasmic system in the same manner as they do in the passive iron system. The temperature coefficient of recovery will be considered below.

Relation between Speed of Transmission and Interval Since Previous Activation.

The total period of recovery in passive iron, *i.e.* the entire interval elapsing between the passage of an activation wave along the wire and the return of the original speed of transmission, is divided into two subperiods: an earlier, during which transmission is limited or decremental, the decrement becoming progressively less and less,[25] and a later in which transmission is unlimited but slower than normal. These two subperiods may be compared with the absolute and the relative refractory periods of living tissues such as nerve and muscle. During the absolute refractory period of nerve (and presumably of other tissues) there is failure of transmission;[26] hence the irritable element fails to respond as a whole to stimulation. In the relative period there is complete response (showing complete transmission), but a stronger stimulus is required than in the completely recovered tissue; apparently this is the reason why transmis-

[24] In Protoplasmic action and nervous action,[5] p. 342, I have given an illustration of how a process consisting of two processes occurring in succession, one with a low, the other with a high Q_{10}, will have a Q_{10} intermediate between the two. The possibility that the electrochemical character of the chief reaction is mainly responsible for the low Q_{10} of transmission was not considered at that time, although in view of the results with the iron model it now seems the most likely explanation.

[25] The numerical measure of decrement is regarded as the reciprocal of the distance travelled by the activation wave before extinction.

[26] Bramwell, J. C., and Lucas, K., *J. Physiol.*, 1911, xlii, 495. Adrian, E. D., *J. Physiol.*, 1915–16, l, 345.

sion during the relative refractory period is slower than normal, as Forbes and his collaborators have recently shown to be the case in nerve.[27] It is sometimes held that no distinction is to be made between the two subperiods beyond that of difference in threshold of stimulation. A strong electrical stimulus, however, is one which is not localized; and if stimulation is initiated at many points of the element, a complete response may result even if transmission is defective. This is also true of passive iron. It seems probable that the difference between the absolute and the relative refractory periods in living tissues is an expression of the difference in the character of the transmission in the two periods, this being decremental in the earlier subperiod and complete in the later, as in the two subperiods of the passive iron model. The time factors of recovery differ greatly in different living tissues, and nerve in particular is characterized by its extremely rapid recovery. In this respect it differs conspicuously from passive iron, but the difference is one of degree rather than of kind.

The main characteristics of the decremental period in passive iron were described in my former paper in this journal.[6] During the succeeding subperiod of complete transmission the speed increases, at first rapidly then slowly, approaching asymptotically a maximum which corresponds to the state of complete recovery. From the general form of the curve of recovery (Fig. 2) it is clear that the increase in speed depends on some progressive change in the system, the rate of this change $\left(\frac{dS}{dt}\right)$ being at any time closely proportional to the distance from the final station. This change can only be a change in the chemical or physical state of the passivating film, leading ultimately to some state of equilibrium which corresponds to the maximal speed of transmission. It is evident that the change in the film is in the general direction of decreased stability, or readier removability. When first laid down the film is relatively resistant to removal by the current of the active-passive circuit; this refractory or resistant quality decreases with time, and the rate of decrease has a high temperature coefficient. Some chemical change in the film is thus indicated. This change is apparently not dependent

[27] Forbes, A., Ray, L. H., and Griffith, F. R., Jr., *Am. J. Physiol.*, 1923, lxvi, 553.

(like the activation wave) on the presence of local circuits; it should also be noted that during its progress the P.D. between the active and the passive areas remains unchanged.[28]

The precise nature of the change in the film during this interval is unknown. Apparently it is of the same qualitative nature (at least in part) as the change occurring during the earlier or decremental subperiod. During the latter period the removal of the film by the electrolytic action of the local circuit appears to be incomplete, *i.e.* the local activation is only partial, hence we may infer that the P. D. between the active and the passive areas is less than in the completely transmissive wire, and falls off progressively as the activation wave travels, finally becoming insufficient to reduce the film. This behavior may indicate that the film when first deposited is relatively thick; if this is true, it seems reasonable to assume that the period of complete transmissivity is one in which the film is approaching its minimal thickness—probably of one molecule. After this period is reached the local reaction at the active-passive boundary has everywhere the same characteristics, and apparently involves the complete breakdown of the film; *i.e.*, the reaction is then of the "all or none" kind, hence the activation wave travels for an indefinite distance.

What is remarkable is that the speed of travel in the completely transmissive period is at first slow and attains its final value by degrees in the manner indicated. Evidently some condition is present at first which impedes or delays transmission; and since transmission is a consequence of the electrochemical reduction of the oxide molecules forming the film,[29] we may infer that at the beginning of the second subperiod a certain proportion of molecules is for some reason relatively inactive or resistant to the reducing action of the current. Why these molecules should be thus resistant is difficult to say. Two possible factors are distance from the

[28] The slight after-positivity observed in a passive wire (connected through a voltmeter with an indifferent electrode) immediately after repassivation (Lillie,[6] p. 115) is an effect of polarization and disappears immediately on stirring.

[29] This is said without prejudice to the view that the final film may consist of a lattice in which molecular limits are not sharply demarcated, as held by Langmuir (Langmuir, I., *Tr. Am. Electrochem. Soc.*, 1916, xxix, 260).

metallic surface and unfavorable orientation. It may be that only those molecules which are attached directly to the iron surface with a constant orientation are fully reactive.[30] On the hypothesis that the film contains at first a certain proportion of unreactive molecules which are changed into reactive molecules by some process following the course of a monomolecular reaction, it is possible to account for the observed behavior of the wires during this period.

Briefly then, we assume that at the beginning of the completely transmissive period the film consists in part of readily reducible or reactive molecules, and in part of refractory or non-reactive molecules; the latter may be regarded as those which are irregularly oriented, or situated beyond a certain distance from the metallic surface. The presence of these molecules retards the reduction of the film, and hence has the effect of delaying transmission; as they decrease in number, *e.g.* by change into active molecules, the speed of transmission increases proportionately. The most reasonable assumption seems to be that inactive molecules are changed into active molecules,[31] and that at any time this change occurs at a rate which is proportional to the number or concentration of the inactive molecules in the film; the change in the speed of transmission follows a parallel course.

Let us assume that at the beginning of the period of complete transmission there is a certain concentration, C_0, of inactive (or

[30] This conception may be brought into relation with the conception of heterogeneous or phase-boundary catalysis recently advocated by Taylor and others; *cf.* Taylor, H. S., Reaction velocity in heterogeneous systems, in Treatise on physical chemistry, New York, 1924, ii, 933; *cf.* 952 *et seq.*, also Chapter XI, Colloid chemistry and contact catalysis, in Bogue, R. H., Theory and application of colloidal behavior, New York, 1924, i, 276. Adsorption means oriented attachment to a surface; in this process the whole electron field of the adsorbed molecule is altered, with corresponding modification of reactivity.

[31] It might be supposed that the inactive molecules are simply *removed; i.e.* dissolved away by the acid. This view, however, seems difficult to reconcile with the fact that the rate at which the speed of transmission increases is much higher in weak than in strong acid, and that in very strong acid (*e.g.* 90 per cent 1.42 HNO_3) the metal may remain incompletely transmissive for many hours (*cf.* Lillie,[6] p. 119). Some peculiarity in the structure or state rather than in the quantity of the film material seems indicated.

impeding) molecules in the film. As these are changed into active molecules the reactivity[32] of the film—hence its rate of reduction and the dependent speed of transmission—increases. The gain of speed is proportional to the increase in active molecules. If the initial concentration of inactive molecules is C_0 and the concentration at the end of time t is C_t, and if the transformation follows the course of a monomolecular reaction, then the usual equation applies: $\frac{1}{t} ln \frac{C_0}{C_t} = K$, K being the velocity constant of the reaction. Or, expressed in exponential form, $C_t = C_0\, e^{-Kt}$. Since the speed of transmission increases with the transformation—the difference from the final speed being at any time proportional to the concentration of inactive molecules remaining—this speed at the time t is a function of $C_0\ (1-e^{-Kt})$; this expression evidently represents the increase in active molecules in the time t.

If we place S_0 as the speed at the beginning of the period, and S_∞ as the final or maximal speed, the total increase in speed is $S_\infty - S_0$; this change corresponds to the complete transformation of the inactive into the active molecules. At any intermediate time t the increase over the initial speed is less and is represented by $(S_\infty - S_0)\ (1 - e^{-Kt})$; this is equivalent to the above expression $C_0\ (1-e^{-Kt})$ which represents the gain in active molecules in time t. Adding the initial speed S_0 to this gain, the total speed, S_t, at time t becomes:

$$S_t = S_0 + (S_\infty - S_0)\ (1-e^{-Kt}),$$

i.e.

$$e^{-Kt} = \frac{S_\infty - S_t}{S_\infty - S_0}$$

or

$$K = \frac{1}{t} \log \frac{S_\infty - S_0}{S_\infty - S_t}.$$

K is the velocity constant for the reaction which determines the return of the film to its fully reactive condition. A difficulty in evaluating K from the above observations is that at the very begin-

[32] As indicated by the rate of chemical change (*i.e.* of reduction) under the influence of the local P.D.

TABLE V.

In this table the values of $K\left(K = \frac{1}{t}\log\frac{S_\infty - S_0}{S_\infty - S_t}\right)$, the velocity constant of the reaction underlying recovery, are evaluated for the four series of Table III. The initial speed, S_0, is regarded as one-third of the final speed, S_∞.

	Recovery interval t. (min.).	Average speed after interval t (cm./sec.). S_t	Difference from final speed. $S_\infty - S_t$	$\frac{1}{t}\log\frac{S_\infty - S_0}{S_\infty - S_t} \times 10^3 = K$
Series I. Temperature 20°.	5	14.6	12.5	32.3
S_0 = 9.0 cm./sec.	6	19.0	8.1	58.0
S_∞ = 27.1 " "	7	18.1	9.0	43.3
	8	20.0	7.1	50.8
	9	22.3	4.8	64.0
	10	22.3	4.8	57.6
	12	25.3	1.8	
	14	27.1		
Average				51.0
Series II. Temperature 15°.	6	10.0	15.3	6.9
S_0 = 8.4 cm./sec.	7	11.5	13.8	12.3
S_∞ = 25.3 " "	8	12.7	12.6	15.9
	9	13.1	12.2	15.9
	10	14.6	10.7	19.8
	12	15.2	10.1	18.5
	14	18.1	7.2	26.5
	16	19.0	6.3	26.8
	20	20.0	5.3	25.2
	25	21.1	4.2	24.1
	30	22.3	3.0	25.0
	45	25.3		
Average from 10 min. on				23.7
Series III. Temperature 10°.	8	10.0	12.3	10.3
S_0 = 7.4 cm./sec.	10	11.5	10.8	13.9
S_∞ = 22.3 " "	12	13.1	9.2	17.7
	14	14.6	7.7	20.5
	16	15.8	6.5	22.5
	20	15.8	6.5	17.9
	25	17.3	5.0	19.1
	30	17.3	5.0	15.9
	45	19.0	3.3	14.5
	60	20.0	2.3	13.5
	90	21.1	1.2	12.3
	∞	22.3		
Average from 12 min. on				17.1

TABLE V—*Concluded.*

	Recovery interval t. (min.).	Average speed after interval t (cm./sec.). S_t	Difference from final speed. $S_\infty - S_t$	$\frac{1}{t} \log \frac{S_\infty - S_0}{S_\infty - S_t} \times 10^2 = K$
Series IV. Temperature 5°.	12	8.8	10.2	6.1
S_0 = 6.3 cm./sec.	16	10.3	8.7	7.9
S_∞ = 19.0 " "	20	10.9	8.1	9.8
	30	12.2	6.7	9.2
	45	14.6	4.4	10.2
	60	15.2	3.8	8.4
	90	17.3	1.7	9.7
	∞	19.0		
Average from 16 min. on.....				9.2

ning of the completely transmissive period transmission is irregular, besides being slow, and comparable measurements are difficult to obtain. Under otherwise similar conditions an activation wave may travel along the whole length of a wire, or may stop abruptly without any apparent cause.[33] Hence it is difficult to assign a definite value to S_0 on the basis of direct observation. The measurements given in Table V were begun within 1 to 3 minutes after complete transmissivity was established; during this interval increase of speed is rapid, especially at the higher temperatures, so that the lowest average speeds given in the table are already considerably higher than the initial speeds. In general, the initial speed may be taken, with a fair approximation, as being about one-third of the final speed, and in Table V the values of K for the four temperatures have been calculated on this assumption.

It will be noticed that in each series the value of K during the first few minutes is decidedly lower than the average. Later it shows a fair approach to constancy, fluctuating irregularly about a mean. Irregularity of transmission is greatest at or near the period of transition between the decremental stage and the completely transmissive stage. Evidently during the decremental stage there is a preponderance of conditions hindering transmission, and

[33] This behavior is illustrated in Tables III to VI of my former paper (*J. Gen. Physiol.*, 1920–21, iii, 107).

it is probable that these conditions persist in part during the early part of the transmissive stage and account for the lower values of K and the greater irregularity then observed. For comparable average values of K at the different temperatures it seems more accurate to omit the earliest measurements, and this is done in the averages given in the fourth column of Table V.

Influence of Temperature on the Recovery of Transmissivity.

From these values of K it is evident that the temperature coefficient of recovery is much higher than that of transmission. The following table gives the approximate Q_{10} determinations as computed from the different values of K at the four temperatures.

Temperature. °C.	K	Q_{10}
5	9	5–10°:3.5+ 5–15°:2.7
10	17	10–15°:2.0
15	24	10–20°:3.0
20	51	15–20°:4.0+

A range of 2.0 to 4.0+ with an average of about 3.0 is shown. The somewhat considerable irregularity of the data does not interfere with this general result, which has been obtained uniformly throughout the present study and in many other observations not included in Tables II, III, and IV.[34]

What is significant is that the temperature coefficient of recovery is high in the passive wire as well as in the living tissue. The duration of the refractory period in muscle and nerve (*i.e.* the period of recovery after a single stimulation) has a high temperature coefficient in all cases, ranging from 2.5 to 3.0 or more; apparently during

[34] It is possible that such irregularities might be decreased by using wires of a more uniform composition. A difficulty in experiments of this kind is that any chance region of a wire may be unfavorable to rapid transmission or regular recovery because of some accidental local peculiarity of structure or composition, such as a condition of "strain" or the presence of an excess of carbon; and if such a wire is used in several determinations the data will be "weighted" in an unforseen manner and unexpected irregularities will appear in the averages.

this period the protoplasmic surface film, altered or broken down during the excitation process, is restored to its original state.[35] It is not necessary—nor indeed possible—to identify the conditions in the living and in the non-living systems in any detail. The two are entirely different in their special chemical composition; what they have in common are certain features of physical structure—in particular the presence of thin impermeable films between the adjacent phases—which determine the conditions under which chemical changes occur. When these changes are of an electrochemical kind, *i.e.* are determined by the action of local currents at surfaces having the properties of electrodes, they are affected by temperature in the same manner as the reactions at the electrodes of a battery. Apparently the essential physicochemical conditions underlying transmission in both systems are of this general kind, hence the low temperature coefficient of the process. On the other hand, changes in the newly formed film, through which the latter is restored to its final or completely reactive state, appear to be independent of electrochemical conditions, and partake of the general nature of reactions between molecules in solution or under analogous conditions. Such reactions have the high temperature coefficients generally regarded as typical of chemical reactions.

Presumably only part of the chemical reactions of living matter are directly influenced by the bioelectric or other currents traversing the protoplasmic system. These reactions, however, are the ones on which the responsiveness to stimulation depends. They are surface reactions—electrode reactions—and determine the electrical sensitivity of living protoplasm. Other reactions, *e.g.* those occurring in the interior of the protoplasmic phases and many enzyme reactions, appear to be unaffected by the passage of electric currents. Distance action, which is so characteristic and indispensable a feature of protoplasmic activity, is a manifestation of electrochemical action and presupposes a special type of structure. This structure is necessarily polyphasic; apparently its essential feature is the presence of two contiguous electrically conducting phases separated

[35] I have reviewed the evidence for the changes in the protoplasmic films during excitation, transmission, and recovery in my recent volume,[5] Chapters 12 to 15.

by a polarizable (*i.e.* semipermeable) surface layer. When the surface is extensive—*e.g.* the surface of an elongated cell or of a filamentous structure such as a nerve fiber—and is covered with a thin electrochemically alterable film, unlimited transmission of the nervous type becomes possible.

Structural Conditions Affecting Speed of Transmission.

The structural as well as the physicochemical conditions in the environment of the passive wire are a factor in the resistance of the

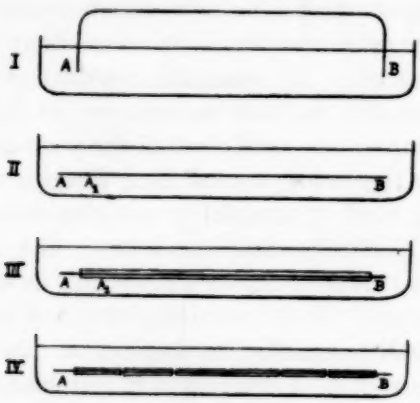

FIG. 3. Arrangements of wires and tubes in trough containing acid.

electrolytic conducting path between its different regions and hence in the immediate effect produced at a distance by a local activation. For example, when the conducting path is narrowed by tubes of non-conducting material the speed of transmission is decreased in the manner already seen. The above rule, $S = k\sqrt{C}$ applies only to the continuous travel of an activation wave along a wire in a tube which is insulated from the acid outside. In the case of a wire inserted (with the ends projecting) through a tube filled with acid and immersed in a larger volume of acid (Fig. 3, III), it may happen that the resistance between the two ends of the wire through the external acid is less than that between the projecting end and a passive area, *e.g.* A_1, situated a short distance inside the tube. In such a case if end A is activated, end B also becomes instantly active,

before the activation wave has visibly penetrated into the tube. This effect is a simple instance of distance action. When any region of the wire becomes active, and hence anodal, all other regions become cathodal; hence any region, however distant, at which the local intensity of current is sufficient, is activated. This intensity at any distant region is determined by the conditions of resistance in the intervening path. If this resistance is low, the activating influence may be transmitted without the continuous passage of an activation wave through the whole distance.

Such "discontinuous" transmission is much more rapid than the continuous wave-like transmission already considered. The following simple experiment is instructive and illustrates the conditions. A bent passive wire held in the air is dipped by its two ends, *A* and *B*, into a vessel of nitric acid (Fig. 3, I). If, then, one end is activated (by touching with zinc or scraping with glass) the other end instantly becomes active. Evidently the activation wave does not travel along the wire through the air; the activation of *A* makes *B* the cathode of the circuit, and the secondary activation at *B* follows instantly since the electrolytic resistance is low. The case of the straight wire inserted through a tube filled with acid and immersed in a large volume of acid (Fig. 3, III) is similar. In this case the current from *A* has two alternative electrolytic paths, one through the general body of acid, the other through the acid inside the tube. The latter path, however, because of its high resistance, is negligible in its effect; it is essentially a shunt diverting a small fraction of current from *B*. Hence the chief secondary electrolysis, *i.e.* activation, is at *B*.

Some curious results follow from these conditions. When a passive wire is enclosed in an interrupted acid-filled tube immersed in acid, as in Fig. 3, IV, and end *A* is activated, the transmission from *A* to *B* may be many times more rapid than along a wire in an insulated continuous tube of the same length. In such an experiment the activation effect appears to leap from one interrupted area or "internode" to the next, and rapidly passes from end to end of the wire. Each internodal passive area of the wire is the chief cathode for the anode at the neighboring activated internode; hence when any internodal area becomes active it instantly activates the next by distance action. Since this effect is repeated at the next internode,

transmission to an indefinite distance becomes possible. An essential condition is that the same structural arrangement should be repeated through the whole distance. The possible distance between internodes is limited by the conditions of resistance, and increases with the specific conductivity and the sectional area of the external body of acid. If the "nodes" or sections of tube are of the appropriate length a saltatory transmission of this type may be extremely rapid, its speed being limited only by the possible internodal distance and the rate at which the passivating film is broken down at the internodes.

The speed of transmission in a wire enclosed by such an interrupted tube is not only greater than in an insulated wire enclosed by a continuous tube of the same length and caliber, but is also greater than in a wire lying free in the acid. The following experiment shows this difference clearly. Two passive wires, each 75 cm. long, were immersed in cold 70 per cent acid (4°). One wire was bare (Fig. 3, II), the other was enclosed by a series of six tubes (of 2.4 mm. caliber) each 10 cm. long and separated from its neighbors by a space of *ca.* 2 mm. (Fig. 3, IV). The wires were activated and a 20 minute interval was allowed for recovery. The average transmission time from end to end in the bare wire (three trials) was then 2.8 seconds; in the tube-enclosed wire it was only a fraction of a second—too brief to measure accurately., The activation could be seen to begin at each internodal area and spread thence in both directions along the tubes; the transmission from each internode to the next appeared almost instantaneous. Many repetitions of this experiment under different conditions of temperature and time of recovery gave the same result. The time required for an activation wave to travel along a continuous insulated tube of 75 cm. length at 4° after 20 minutes recovery is 8 to 10 seconds.

It may seem surprising at first sight that the transmission from *A* to *B* is not equally rapid in the three arrangements II, III, and IV of Fig. 3, since the resistance through the external body of acid is the same in all. The distribution of the entrance and exit of current along the wire—and hence the chemical effect at its different regions —is, however, characteristically modified by the presence of the tubes. The enclosing tube limits the effective area of cathode in

the immediate neighborhood of an active (*e.g.* internodal) area. Since according to Faraday's law the quantity of electrochemical change in the cathodal area is equal to that in the anodal area, it is clear that the effective cathodal area cannot have a greater extent than one which corresponds to the reaction occurring in the anodal area. In the bare wire the region immediately adjoining the active area furnishes the necessary area of cathode; hence beyond this area there is no chemical effect. In the presence of the tube, on the other hand, the cathodal area is mainly furnished by the wire *beyond* the tube. Hence, since the anodal and the cathodal reactions are necessarily simultaneous, the reaction in the area B of wire III begins at the same time as the reaction in the area A_1, in wire II. Transmission to a distant area is correspondingly rapid. It is evident that the conditions in wire IV constitute simply a serial repetition of those in wire III.

Whether conditions analogous to those just described enter in the case of nerve and other transmitting structures in living organisms is difficult to determine experimentally, but seems not improbable. We observe, for example, that in the most rapidly conducting protoplasmic tracts known, the medullated nerves of vertebrates, the conducting element (axone) is enclosed by a tubular sheath of apparently high electrical resistance, the medullary sheath, which is constricted or interrupted at regular intervals. The medullated nerve transmits impulses at about ten times the velocity of the non-medullated nerve, in which, except for the absence of the segmented sheath, the structure is similar. In the frog the sympathetic tracts to the skin conduct at *ca.* 2 meters per second (at 17°)[33] as compared with *ca.* 20 meters for the medullated nerves. It is possible that a a distance action effect, acting from internode to internode (as in the model above) is a factor in this high speed of transmission. The electrical resistance between the surface of the axone and the surrounding medium may be assumed to be relatively low at the constrictions or internodes; diffusing substances (dyes) enter most readily at those regions, and the same is presumably true of ions. At least such a possibility must be considered; for in the physical

[33] Yamada, S., *Arch. ges. Physiol.*, 1923, cc, 221.

sense—if transmission is an effect of secondary stimulation by the currents of local circuits—the conditions in the model and in the nerve are of the same general kind. That the chief regions of entrance and exit of electrical currents transversing living tissues and organisms are determined by structural conditions, and that these regions are definitely localized in many cases, have long been accepted beliefs. The older conception of physiological anodes and cathodes should be extended so as to apply to the finer elements of structure and function, possibly even to single cells.

SUMMARY.

1. The speed of transmission of the activation wave along passive iron wires enclosed in glass tubes containing dilute (70 per cent) nitric acid increases with the conductivity (sectional area) of the column of electrolyte but at a slower rate. The speed is closely proportional to the square root of the conductivity ($S = k\sqrt{C}$). The reasons for this relationship are discussed and an explanation is proposed.

2. The recovery of transmissivity after the passage of an activation wave is gradual and follows a characteristic course. After an interval of partial or decremental transmission (having a high temperature coefficient and lasting several minutes at 20°), the wire recovers its power of transmitting an activation wave for an indefinite distance. In such a recovered wire the speed of transmission is at first slow and increases by degrees up to a maximum, the increase following a curve apparently of the type $v_t = v_0 (1 - e^{-kt})$. The approximate time required to attain this maximum (corresponding to complete recovery) at the different temperatures is 15 to 20 minutes at 20°, 30 to 45 minutes at 15°, *ca.* 60 minutes at 10°, and 90 minutes or more at 5°.

3. The character of the curve of recovery (the curve relating speed of transmission to interval since previous activation) agrees with the assumption that the increase in speed depends on a progressive chemical change in the molecules forming the passivating film, this change involving the transformation of (relatively) nonreactive into reactive molecules and following the course of a monomolecular reaction.

4. The temperature coefficient of the speed of transmission (between 5° and 20°) is low, of the order Q_{10} = 1.3 to 1.6. That of the rate of recovery, on the contrary, is high (Q_{10} = *ca.* 3). The parallel to the conditions in nerve and other transmitting protoplasmic systems is pointed out and discussed.

5. Passive wires enclosed in acid-containing continuous and interrupted glass tubes immersed in a large volume of acid exhibit characteristic phenomena of distance action; under appropriate conditions the velocity of transmission of the activating influence between different areas may thus be greatly increased. Characteristic instances are cited and some possible physiological parallels are pointed out.

SYMBIOSIS BETWEEN CHLORELLA SP. AND AZOTOBACTER CHROOCOCCUM AND NITROGEN FIXATION.

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Alfred Koch has given considerable attention to the subject of nitrogen fixation by bacteria as resulting from their symbiosis with algæ. Many others have also investigated various features of this problem. In spite of all this there are numerous questions which remain unsolved. The use of soil cultures for such studies is objectionable on account of the soil's complexity, its variety of flora and fauna, and the impossibility of observing the organisms while they are at work; and yet the soil culture method has been the one most used by investigators heretofore.

A fortunate accident threw into our hands a species of *Chlorella* which we are using in some physiological experiments. The facile growth of this organism under ordinary laboratory conditions in tap water, in distilled water, and in weak culture solutions offered a ready means for experimentation on the question of a possible symbiosis with *Azotobacter*. This strain of *Chlorella* develops in solution cultures a beautiful green granular growth and with proper light conditions covers the walls of the culture flask completely. It is a single celled organism, somewhat elliptical in shape, and not much more than twice as large as the large cells of *Azotobacter chroococcum*. Its cell wall and chloroplasts are very distinct and the structure of the cell unusually clear under the microscope. The organism probably comes from the tap water of the city of Berkeley, but it is undoubtedly a common inhabitant of surface soils. Some unusually striking physiological peculiarities of this strain of *Chlorella* will be described in a later publication from this laboratory.

The *Azotobacter* culture used in this investigation was a pure culture of *Azotobacter chroococcum* (Madera strain) which was isolated from a California soil.

Technique and Results of the Experiment.

A culture of *Chlorella* which had been growing under greenhouse conditions for a number of weeks in an inorganic culture solution in an Erlenmeyer flask stoppered with cotton was transferred to a large graduated cylinder and made up with sterile tap water to a volume of 600 cc. After thorough agitation 50 cc. portions of this diluted culture were measured out into 300 cc. Erlenmeyer flasks. This great dilution of the culture of *Chlorella* should make the total amount of dissolved matter comparable to that of the tap water of the city of Berkeley. This tap water has a total concentration of inorganic salts of approximately 400 parts per 1,000,000. It contains about 65 parts per 1,000,000 of Ca and Mg combined, and about 90 parts per 1,000,000 of Na. The total loss on ignition in the residue of this tap water is about 100 parts per 1,000,000. The organic matter in the culture solution made up as described above would come from the small amount dissolved in the tap water and from the dead cells of *Chlorella* which had accumulated in the original culture. This must, of course, constitute a very slight source of organic matter. *Azotobacter chroococcum* alone, without the presence of living cells of *Chlorella*, in such a culture solution can fix practically no nitrogen. The question, therefore, remains whether it can fix nitrogen when it has with it living cells of *Chlorella* in the same culture solution. No carbohydrate was added to any of the culture solutions used in this experiment.

To every 50 cc. culture prepared as above described 1 cc. of a suspension of a fresh culture of *Azotobacter chroococcum* was added. Six of the mixed cultures were analyzed immediately for total nitrogen by the modified Gunning method. The other six cultures were allowed to incubate in the laboratory in fairly subdued light for a period of a month. The flasks were plugged with cotton to keep out other organisms and dust. In addition to the mixed cultures of *Chlorella* and *Azotobacter* we prepared several similar 50 cc. cultures of *Chlorella* alone which were incubated side by side with the mixed cultures to serve as a control. After a few days of incubation we noticed a marked difference between the *Chlorella* cultures with *Azotobacter* and those without *Azotobacter*. The pure culture of *Chlorella* while normal in appearance was light green in color and seemed to be growing slowly. The mixed culture, on the other hand, was beautiful emerald green and

was growing rapidly, producing a characteristic granular growth. The discrepancy between the cultures of the *Chlorella* alone and those of *Chlorella* with *Azotobacter* was maintained and even grew more marked as the period of incubation progressed. After about 1 month incubation the cultures were analyzed for total nitrogen with the results indicated in Table I.

The data in Table I show clearly that while the absolute gains of nitrogen in the mixed cultures of *Chlorella* and *Azotobacter* were small, the relative gains are large. Moreover, they show that *Azotobacter* is able to use the carbohydrate synthesized by *Chlorella* as a source of energy for nitrogen fixation, since every culture gained in nitrogen. The highest figure for total nitrogen in the six control cultures is

TABLE I.

Total N in Cultures of Chlorella with Azotobacter.

Culture No.	N found.	N in control.	N fixed.
	mg.	*mg.*	*mg.*
1	1.40	0.70	0.70
2	1.26	0.98	0.28
3	1.54	0.98	0.56
4	1.12	0.84	0.28
5	1.26	0.64	0.62
6	1.26	0.64	0.62
Average.....	1.31	0.80	0.51

appreciably lower than the lowest figure for the incubated mixed cultures. When one remembers in addition how small the amount of dry matter is in every culture it is remarkable that on the average about 0.5 mg. of nitrogen was fixed. This indicates a high efficiency of the process under the conditions in question.

Our results in this experiment are preliminary in nature. We hope to carry on futher experiments along this line to discover more about the process of nitrogen fixation in the symbiotic relationship into which *Chlorella* and *Azotobacter* seem to enter so effectively. It would also seem that attractive possibilities exist for practical applications of these studies. In the case of sandy soils particularly it might be possible to inoculate with *Chlorella* and *Azotobacter* to increase the soil nitrogen supply cheaply and effectively.

THE ACID AGGLUTINATION OF MIXTURES OF OPPOSITELY CHARGED BACTERIAL CELLS.

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It is quite generally supposed that suspensions of colloidal particles with opposite electric charges attract one another and that if the charge is the same, they repel. This theory has been suggested by Northrop and De Kruif as part of the mechanism of bacterial agglutination.[1] In their experiments with *Bacterium lepisepticum*, the bacillus of rabbit septicemia, they found the original strain, Type D,[2] to be very stable in suspension and to possess a high negative charge. Type G, the dissociated, so called mutant form, had a very low negative charge and was less stable. Other conditions being the same, agglutination of both types was said to occur when the potential was reduced to about 15 millivolts.

If it is true that bacteria are held apart by similar electric charges, they should be brought together by opposite charges. That is, two types of bacterial cells in a hydrogen ion concentration such that the charge of one is positive and of the other, negative, should agglutinate. This is the usual result with ordinary colloidal suspensions but does not seem to have been recorded for the case of oppositely charged bacteria. Experiments to test this theory follow.

Three cultures of *Bacterium lepisepticum*, obtained from different rabbits, were employed. Pure strains of Types D and G were grown in extract broth containing a small amount of hemolyzed blood.[3] They were then washed carefully four times[1]

[1] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

[2] De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.

[3] Webster, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 139.

TABLE I.

Agglutination of Culture 103, Types D and G.

D	G	Na lactate buffers.									
		pH 4.7	pH 4.5	pH 4.1	pH 3.8	pH 3.5	pH 3.3	pH 3.0	pH 2.7	pH 2.4	Cont.
per cent	*per cent*										
100	—	—	—	—	+	C	C	C	++	+	—
80	20	—	—	—	C	C	C	C	++	+	—
60	40	—	+	C	C	C	C	C	++	+	—
40	60	+	C	C	C	C	C	++	+	±	—
20	80	C	C	C	C	C	++	+	—	—	—
—	100	C	C	C	+	—	—	—	—	—	—

TABLE II.

Agglutination of Culture 115, Types D and G.

D	G	Na lactate buffers.									
		pH 4.7	pH 4.5	pH 4.1	pH 3.8	pH 3.5	pH 3.3	pH 3.0	pH 2.7	pH 2.4	Cont.
per cent	*per cent*										
100	—	—	—	—	—	C	C	C	C	+	—
80	20	—	—	—	++	C	C	C	++	—	—
60	40	—	—	C	C	C	C	C	++	+	—
40	60	—	C	C	C	C	C	++	+	—	—
20	80	C	C	C	C	++	+	—	—	—	—
—	100	C	C	++	—	—	—	—	—	—	—

TABLE III.

Agglutination of the Rivers Strain, Types D and G.

D	G	Na lactate buffers.									
		pH 4.7	pH 4.5	pH 4.1	pH 3.8	pH 3.5	pH 3.3	pH 3.0	pH 2.7	pH 2.4	Cont.
per cent	*per cent*										
100	—	—	—	—	+	C	C	C	++	+	—
80	20	—	—	+	C	C	C	C	C	±	—
60	40	—	±	C	C	C	C	C	++	+	—
40	60	±	C	C	C	C	C	++	±	—	—
20	80	C	C	C	C	++	—	—	—	—	—
—	100	±	++	C	—	—	—	—	—	—	—

and finally suspended in distilled water so that similar degrees of turbidity, about 500,000,000 organisms per cc., were obtained.

These two suspensions were combined in various proportions and added in 1 cc. amounts to tubes containing a like volume of sodium lactate buffer at pH concentrations ranging from 2.4 to 4.7.[4] The tubes were then placed in a water bath at 55° and examined at 6 and 18 hours.

The results for the three cultures were similar (Tables I, II, and III). The agglutination zone of the Rivers strain, No. 103, and No. 115, Type D, was pH 2.4 to 3.8; that of the G types ranged from 4.1 to 4.7. And it may be seen that while the agglutination zones of pure D and G scarcely overlapped, a mixture of the two types in various proportions resulted in complete flocculation in the intermediate zone, pH 3.8 to 4.1.

Evidently, then, a hydrogen ion concentration at which Type D bacteria are negatively and the G forms positively charged furnishes the proper conditions for optimum flocculation of mixtures of these bacteria. This behavior of oppositely charged bacteria in suspension is the same as that of similar unorganized colloidal particles and supports the theory that bacteria with like charges are repelled.

Thanks are due to Dr. John H. Northrop for helpful suggestions and to C. G. Burn for assistance with the experiments.

[4] De Kruif, P. H., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 34.

THE GROWTH OF DUCKWEEDS IN MINERAL NUTRIENT SOLUTIONS WITH AND WITHOUT ORGANIC EXTRACTS.

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(Accepted for publication, January 14, 1925.)

In recent years doubt has been thrown upon the assertion that green plants are able to obtain all their raw materials from the atmosphere and inorganic matter of the soil. Experiments that have been reported within the last 10 years suggest that possibly still another factor in nutrition, besides the carbon dioxide in the atmosphere and inorganic salts in the soil solution, is necessary for the complete growth of green plants. This factor is believed to be in the form of organic substances. Bottomley (2–4) believed minute amounts of organic matter to be essential to the continued growth of green plants and gave them the name "auximones."

Bottomley grew *Lemna minor* (5–7) in Detmer's mineral nutrient solution with and without extracts of a specially treated peat which he called "bacterized peat" (1, 3). From his results he concluded that *Lemna minor* cannot grow for any length of time in a pure mineral culture solution, and that the presence of soluble organic matter (auximones) was essential for complete growth.

Miss Mockeridge (13), working with Bottomley, studied the effect of extracts of leaf mold, soil, fresh and well rotted stable manure, and of bacterized peat on the growth of *Lemna major*, using Knop's as the basal solution. The addition of the extracts caused an increase in number of plants produced, and in dry weight per plant, over the controls. The controls (Knop's solution) decreased in dry weight per plant. Her results were in substantial agreement with Bottomley's.

Mendiola (12), studying clonal variations in *Lemna minor*, stated that his plants would grow in a modified Pfeffer's solution, and disagreed with Bottomley's statement.

Duggar (10), in noting the effect of excision of cotyledons upon the growth of peas, believed that the cotyledons might contain a "vitamine requisite" for the vigorous development of the plant.

Robbins (14) grew excised root tips under sterile conditions, and found that the addition of small amounts of peptone or of autolyzed yeast to a Pfeffer's solution plus glucose considerably lengthened the period of growth of the tips. It is stated that the results would seem to be best explained by assuming that the seedling root contains some substance or substances derived from the grain which are not supplied by the mineral salts, glucose, free oxygen, and water.

Recently Clark and Roller (8, 9) have published results of experiments with *Lemna major*. They do not believe auximones are essential for the growth and reproduction of green plants.

Methods.

Since Bottomley and Mockeridge claimed that green plants require both mineral nutrients and organic accessory food for normal growth, it was decided first to attempt an experiment similar to the ones they performed. Their chief work was done with *Lemna major* and *Lemna minor*. Since these species were not available at the time it was decided to use another of the Lemnaceæ, *Spirodela polyrhiza* (L.) Schleiden (*Lemna polyrhiza* L.).

In general the methods employed by Bottomley and Mockeridge in growing *Lemna* were followed in preparing the cultures of *Spirodela*. 225 cc. of the nutrient solution were placed in cleaned tumblers of about 250 cc. capacity. At first the tumblers were wrapped separately with black paper, to cut off illumination from the sides. This was an inconvenience during washing, so a box containing as many compartments as desired, usually fifteen, was constructed of black cardboard. The compartments were just large enough to hold the tumblers, and exactly as deep as their height. This arrangement is believed to be efficient in shading from light, and has the advantage of allowing the tumblers to be cleaned rapidly without removal and replacement of paper. In all experiments the plants were protected from falling dust by placing a sheet of glass about 3 cm. above the solutions. Ten plants were placed in each culture at the beginning of an experiment. The plants were washed in distilled water and transferred to fresh solutions at intervals ranging usually from 4 to 7 days, at which time the plants were counted. Whenever dead leaves were found their number was recorded. As the number of plants increased so that the cultures became crowded, either half the number, or else all except a certain number were removed. Such

discarded plants were used to obtain dry or green weights. The total number of plants that would have been produced, had none been discarded, was calculated. The cultures were kept in the laboratory, near windows exposed to the south or east. Since ordinary distilled water was found to be less favorable for growth, redistilled or "conductivity" water was used in preparing the nutrient solutions and extracts. This water was prepared by distilling from acidified potassium permanganate, then from alkaline potassium permanganate, using a Pyrex glass still. The water so prepared was nearly neutral in reaction.

The mineral nutrient solutions were prepared as follows:

Detmer's Solution.	gm.	*Knop's Solution (Modified).*	gm.
KNO_3	23.3	KNO_3	3.0
K_2HPO_4	5.0	KH_2PO_4	3.0
$MgSO_4 \cdot 7H_2O$	5.0	$MgSO_4 \cdot 7H_2O$	3.0
NaCl	5.0	$Ca(NO_3)_2 \cdot 4H_2O$	9.0
$CaSO_4 \cdot 2H_2O$	16.7	$FeCl_3$	0.72
$FeCl_3$	trace.	Redistilled water	18 liters.
Redistilled water	10 liters.		

Knop's was prepared from three stock solutions, one containing the first three salts, the second the calcium nitrate, and the third the ferric chloride. The salts used were Merck's Blue Label, Baker's Analyzed, or salts of similar quality.

The organic extracts were prepared in the following manner. *$NaHCO_3$ Extract of Raw Peat, or Bottomley's "Nucleic Acid Derivative of Raw Peat"* (7).—A weighed quantity of raw peat was extracted with successive portions of a 1 per cent solution of $NaHCO_3$ until the extract was almost colorless. The combined extracts were neutralized with HCl, and a few drops of chloroform were added to prevent bacterial growth. Before use the chloroform was removed by heating over a water bath. The peat used was black and well decomposed.

Water Extract of Autolyzed Yeast.—5 gm. of autolyzed yeast (Fleischmann's) and 250 cc. of distilled water were heated to the boiling point, then filtered through the same filter paper until clear. The extract was preserved with chloroform.

Spirodela polyrhiza Grown in Dilute Knop's Solution and in Dilute Detmer's Solution.

Experiment 1.—Preliminary experiments indicated that both Detmer's and the modified Knop's solutions were unfavorable to the growth of *Spirodela polyrhiza*. To determine what effect reducing the concentration of salts in these solutions might have, Knop's solution was diluted to 5, 10, 50, and 150 times its volume with distilled water. Three cultures of ten plants each were set up for each dilution. The dilutions of Knop's solution are hereafter abbreviated as follows: Knop/5, Knop/10, Knop/50, and Knop/150. The plants were transferred to fresh solutions about once a week, the experiment extending over a period of 33 days (July 7 to Aug. 9, 1922).

TABLE I.

Spirodela Growing in Varying Dilutions of Knop's and Detmer's Solutions.

Solution.	Original No. of plants.	No. of plants at end of experiment.	No. of dead leaves.	Dry weight of all plants.	Dry weight per 100 plants.
				mg.	*mg.*
Knop's	30	90	263	9.2	10.2
Knop/5	30	95	123	45.0	47.4
Knop/10	30	98	111	53.9	55.0
Knop/50	30	105	189	28.2	26.8
Knop/150	30	69	220	8.9	12.9
Detmer's	30	64	123	—	—
Detmer/5	30	73	81	—	—
Detmer/10	30	78	74	—	—
Detmer/50	30	66	69	—	—
Detmer/150	30	61	68	—	—

A similar experiment, using varying dilutions of Detmer's solution, but extending over a period of only 16 days, was carried out at the same time. The data are given in Table I.

Results.—In the Knop's solution series the greatest amount of growth, as measured by dry weight, occurred in Knop/10. The greatest number of individual plants occurred in the Knop/50, but the average size of these plants was below that of the plants growing in Knop/10.

The results for the Detmer's solution series indicate again the favorable effect of dilution, since more plants were produced in the Detmer/10 than in any other of the dilutions.

In both sets of solutions it is evident that too great dilution is unfavorable, the conditions of the plants approaching that of plants grown in distilled water.

Spirodela polyrhiza Grown in Dilute Knop's Solution and in Pond Water, with and without the Addition of Organic Extracts.

Experiment 2.—Although Experiment 1 described above had not been completed at the time this one was begun, the indications pointed to Knop's solution diluted to 10 times its volume (Knop/10) as the most favorable one in the series for growth of *Spirodela*. For this reason a set of cultures was prepared to determine the effect of extracts of autolyzed yeast and of peat upon *Spirodela* growing in Knop/10. For comparison a similar series was prepared, using the

TABLE II.

Spirodela Growing in Knop's Solution and in Pond Water, with and without Organic Extracts.

No.	Solution.	No. of plants at beginning of experiment.	No. of plants after 23 days.	No. of dead leaves.	Total dry weight.	Dry weight per 100 plants.
					mg.	*mg.*
1– 3	Knop/10 only.	30	90	98	26.2	29.1
4– 6	Knop/10 plus yeast extract.	30	183	25	69.6	38.0
7– 9	Knop/10 " peat "	30	117	34	65.0	55.5
10–12	Pond water only.	30	72	68	47.2	65.5
13–15	" " plus yeast extract.	30	246	48	68.2	27.7
16–18	" " " peat "	30	98	69	59.2	60.4

pond water of the plant's natural habitat as the basal solution. The following cultures were therefore prepared after the usual manner.

Nos. 1, 2, 3. Knop/10 only.

Nos. 4, 5, 6. Knop/10, every liter of which contained 20 cc. distilled water having the soluble material of 0.4 gm. autolyzed yeast, obtained by boiling the autolyzed yeast with 50 times its weight of distilled water and filtering.

Nos. 7, 8, 9. Knop/10, every liter of which contained 66 cc. water having the soluble material of 2.5 gm. raw peat, obtained by extracting with 1 per cent $NaHCO_3$ solution, as outlined under Methods.

Nos. 10, 11, 12. Filtered pond water.

Nos. 13, 14, 15. Filtered pond water, containing a similar and like amount of autolyzed yeast extract as Nos. 4, 5, and 6.

Nos. 16, 17, 18. Filtered pond water, containing a similar and like amount of peat extract as Nos. 7, 8, and 9.

The condensed data are presented in Table II. Counts were made and the solutions were renewed after 4, 8, 12, 16, and 23 days, after which the dry weights were obtained. The period of the experiment was July 18 to Aug. 10, 1922.

Results.—The addition of small quantities of autolyzed yeast extract and of peat extract to both dilute Knop's solution and pond water caused an increase in the number of plants of from 1.4 to 3.4 times.

The addition of these extracts increased the total dry weight of all the plants in both dilute Knop's solution and in pond water from 1.3 to 2.6 times.

The average dry weight per plant, however, was increased by the addition of these extracts only in the case of the dilute Knop's solution.

Growth of Spirodela polyrhiza and of Lemna valdiviana in Mineral Nutrient Solution without Organic Extracts.

Experiment 3.—On November 4, 1922, ten plants of *Spirodela polyrhiza* were placed in the dilute Knop's solution used in Experiment 2 (Knop/10). The plants available apparently had completed their season's growth. Vegetative growth seemed to have ceased, and dormant winter fronds were being produced in abundance.[1] After 2 weeks in the nutrient solution all plants had begun to grow actively, and after 4 weeks the production of winter fronds had practically ceased.

The plants were kept in battery jar aquaria or in tumblers, shaded on the sides by black paper. They were transferred to new solutions at intervals usually of 1 or 2 weeks. As the plants increased in number the surplus was discarded. Data on weight of plants or percentage increases in number of plants were not obtained for the entire period of the experiment, but data collected over a period from Mar. 31 to Aug. 4, 1923, showed that the rate of increase in number of plants per week ranged from 9 per cent during the 1st week of April to over 100 per cent during June and July.

The plants of this same culture have now been kept in the dilute Knop's solution for a period of over 26 months (Jan. 4, 1925), without the addition of any organic matter except that which might have been introduced through the chemicals used, or through the dust particles from the air falling into the solutions.

On Mar. 31, 1923, ten plants of *Lemna valdiviana* Phillipi were placed in the dilute Knop's solution (Knop/10). These have been grown successfully in this

[1] By October vegetative reproduction in *Spirodela* practically ceases under conditions of the local natural habitat, and the plants then produce an abundance of small dormant winter fronds. These later become detached, sink to the bottom of the pond, rise again to the surface the following spring, and germinate.

solution for over 21 months (Jan., 1925) without any apparent reduction in size or rate of growth.

Results.—Even though no comparison has been made with the growth of similar plants in pond water, the experiment shows that the two species mentioned are able to grow and multiply normally over long periods of time in a mineral nutrient solution devoid of organic extracts.

DISCUSSION.

Since none of the species of Lemnaceæ used in this investigation was the same as those used by Bottomley (5) or by Miss Mockeridge (13), strict comparison with their results cannot be made. But attention may be called to the cases where the results obtained with the nearly related species, *Spirodela polyrhiza* and *Lemna valdiviana*, agree or disagree with the conclusions of these authors.

The solutions employed in preliminary experiments, Detmer's and a modified Knop's, were unfavorable to the growth of *Spirodela polyrhiza*. Of the two, Detmer's solution was the more toxic. When a small quantity of an extract of autolyzed yeast was added to Knop's solution it was considerably improved, resulting in growth far in excess of the mineral solution alone, and also in excess of the growth in pond water obtained from the plant's natural habitat. The question arises as to how the autolyzed yeast can improve an unfavorable mineral culture medium. According to Bottomley and Mockeridge the small quantities of organic matter furnished auximones, which supplied a deficiency in the mineral solution. However, as pointed out recently by Clark and Roller (9) the reason for poor growth may have been the unsuitability of the mineral solution. In order to produce a suitable solution they followed the general methods of Shive (15) and others (11). The author used a simpler method with success, that of diluting the unfavorable Knop's solution with distilled water. Such a dilute Knop's solution has supported growth of *Spirodela polyrhiza* for a period of 26 months, and of *Lemna valdiviana* for a period of over 21 months, without resulting in any permanent decrease in size of plants or in rate of reproduction. In fact the *Spirodela* plants at the end of 23 months appeared larger and more healthy in the mineral solution than similar

plants growing in the pond from which the original plants for the experiment were obtained. The reason for this improvement of Knop's solution by dilution is a subject under further investigation. The important point is the fact that a purely mineral solution can be prepared which will support growth and reproduction of species of Lemnaceæ over a long period of time. The necessity of organic accessory foods or auximones for the continued growth of green plants, Lemnaceæ at least, cannot be accepted as an established fact.[2]

The question now arises as to the manner in which small quantities of autolyzed yeast extract improved the unfavorable Knop's solution. As pointed out by Robbins (14), who used a similar extract in prolonging the growth of excised root tips, the autolyzed yeast may supply substances which balance the solution. It is known that yeast cells often have a high ash content (Fleischmann's 8.74 per cent of dry weight), and it is possible that the benefit derived from the addition of the yeast extract may be due to the balancing effect of some substance or substances which it contains.[3] The view might also be taken that the yeast furnishes an additional food supply which is not necessarily required by the plant but which, when added, results in a stimulation of growth and reproduction. This view is held by Clark (8), who suggests that the auximones may be of the same nature as bios in the reproduction of yeast. However, the benefit derived from the addition of extracts of organic matter to unfavorable mineral solutions may be due to another cause or to a combination of other factors.

Experiments 1 and 2, and especially Experiment 3, seem to show that the dilute Knop's solution *alone* is a favorable culture medium for the growth of *Spirodela* and *Lemna*. However, Experiment 2 shows that the growth of *Spirodela* in this *favorable* solution can also

[2] The above conclusion is open to this criticism: auximones might have been added to the mineral solution as a contamination of the chemicals used, they might have entered as particles of dust from the atmosphere, or they might have been supplied by the bacteria always present in such cultures. The frequent transfer of the plants to fresh solution, however, prevented excessive bacterial contamination.

[3] A later experiment has shown that the water-soluble material of 0.4 gm. autolyzed yeast plus a liter of distilled water cannot support growth of *Spirodela* for much more than 4 weeks.

be stimulated by the addition of yeast or of peat extracts, although not to such an extent as in the more concentrated solutions. Whether a purely mineral solution can be prepared which is not improved by the addition of extracts of organic matter cannot as yet be definitely stated. A number of preliminary experiments seem to indicate that at least one species of Lemnaceæ is retarded in growth by the addition of a yeast extract, and also that the stimulating effect of a peat extract on *Spirodela* is diminished after a number of months, resulting in an actual retardation of growth as compared with controls in a mineral solution.

SUMMARY.

1. Detmer's solution and a modified Knop's solution are unfavorable culture media for the growth of *Spirodela polyrhiza*.

2. When the modified Knop's solution was diluted to 10 times its volume, *Spirodela polyrhiza* and *Lemna valdiviana* grew and reproduced for periods of 26 months and 21 months, respectively.

3. Growth in the *dilute* Knop's solution, which alone can support the growth of *Spirodela* indefinitely, was considerably stimulated over a period of 23 days by adding to every liter the water-soluble material of 0.4 gm. autolyzed yeast, or the material of 2.5 gm. peat soluble in a 1 per cent solution of $NaHCO_3$.

4. The nature of the stimulus or of the protection afforded by the organic material is as yet unknown.

5. The necessity of organic accessory foods (auximones) in the nutrition of green plants cannot be accepted as an established fact.

The experimental work reported here was done in part in the Department of Botany of the University of Missouri, Columbia, Mo., and in part in the Department of Biology of Junior College of Kansas City, Kansas City, Mo.

Acknowledgement is due to Professor W. J. Robbins of the University of Missouri, who suggested the problem and on occasions offered helpful advice, and to Dr. W. E. Maneval for aid in preparing the manuscript.

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METABOLISM DURING EMBRYONIC AND METAMORPHIC DEVELOPMENT OF INSECTS.

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PLATE 4.

(Accepted for publication, January 15, 1925.)

Insects, because of their abundance and wide variety in form and structure, have become more widely recognized in recent years as excellent material for the study of many physiological problems. Morphologists have intensively studied the developmental processes of insects, while relatively few physiologists have investigated such phenomena as histolysis and histogenesis occurring during pupal development. The most notable physiological work on these subjects seems to have been done by Farkas (6), Krogh (10), Tangl (13), and Weinland (14).

Weinland, as early as 1906, in his studies on *Calliphora vomitoria* (blow-fly), utilized the gaseous exchange of the organism as a basis for estimating the chemical changes occurring during histolysis and metamorphosis. He found that the larvæ liberated ammonia, whereas in the pupæ uric acid was produced. This clearly indicates, he states, a change in the chemical process in the development of the insect. His experiments with metabolism in general indicate a markedly low CO_2 output at the beginning or early stages of pupation, which is followed by a period of rest. In later stages, however, an increase in the gaseous exchange of the organism took place.

Tangl, in 1909, obtained results from metabolism experiments on *Bombyx mori* (silkworm) similar in many respects to those of Weinland. Extending his researches in other directions, however, and comparing his own results on the larvæ and pupæ of *Bombyx mori* with those obtained by Farkas on the embryonic development of the same form, Tangl was led to enunciate his energetic theory. This theory postulates that the amount of energy required in the process of embryonic development is greater and the rate of energy change more intense than is required for metamorphosis.

To the author it seems reasonable to suppose that the building and multiplication of cells from the relatively inert, non-living substance of the yolk, with its large store of unorganized material and infinitesimal amount of protoplasm, should require more energy than the process of reconstruction from histolytic products in metamorphosis, since this latter process is largely a matter of growth of imaginal tissues.

The histological investigations of Weismann (15) and others have shown that the larvæ at birth possess two kinds of tissues, larval and imaginal. During embryonic and postembryonic development, larval tissues are dominant, whereas imaginal tissues are greatly retarded in their development. It is possible to conceive of the existence of such dominance as long as the larval tissues and organs are in a functioning condition. At the termination of the larval life, histolysis and disintegration of the larval tissues and organs seem to produce a condition favorable for the development and growth of the imaginal tissues.

The causes that initiate histolysis do not seem to be clear. Weinland, however, as stated above, has pointed out that during the larval life of *Calliphora vomitoria* ammonia is produced, whereas during pupal development uric acid is formed. This condition led Weinland to assume that the chemical changes thus produced are the most significant. Mathews (12), however, states that while in carnivorous animals the urine is acid in nature, in herbivorous animals the urine may be distinctly alkaline. Bishop (3) has recently found that an acid medium at the beginning of pupation is favorable to autolysis (*in vitro*) in bee larvæ. Experiments performed by the author indicate that changes in pH from a neutral to an acid condition occur in the larval and pupal fluids of certain insects during histolysis and pupal development. Changes in pH from an acid to a neutral condition have also been observed to take place with some species from early to late embryonic development. During embryonic development, however, no histolysis occurs. The question, therefore, naturally arises, is the change from neutrality to acidity responsible for histolysis, or a result?

The object of the present paper is to describe the results from studies of the rates of respiratory metabolism and other phenomena during the embryonic and metamorphic development of certain insects.

Material and Method.

The material used consisted exclusively of insect eggs and pupæ derived from different species of five orders.

The following species of insects were used.

Leptinotarsa decemlineata Say (potato beetle).
Crioceris asparagi L. (asparagus beetle).
Epilachna borealis Fab. (squash ladybeetle).
Hippodamia convergens Guer. (convergent ladybeetle).
Cotinis nitida L. (green June beetle).
Popillia japonica Newm. (Japanese beetle).
Ancylis comptana Frohl. (strawberry leaf-roller).
Macrocentrus ancylivora Rohw. (hymenopterous parasite).
Hylemyia cilicrura Rond. (seed-corn maggot).
Anasa tristis DeG. (squash bug).

That the respiratory exchange of an organism is one of the best criteria of metabolic activity seems to be an established fact. In the present experiments, therefore, the rates of metabolism as measured by the CO_2 output and the oxygen intake have been determined.

Two types of apparatus were found useful. First, the manometer described by Krogh (10) (Plate 4). This manometer has been slightly improved by Dr. Bodine of the University of Pennsylvania. The improvements consist in the elimination of all rubber connections, thus rendering the instrument especially reliable and accurate for metabolism experiments on insects. For detailed descriptions and methods of calculation of the results, the reader is referred to the original article by Krogh (10).

The second type of apparatus for measuring the rates of CO_2 output of small organisms is essentially the Haas colorimetric method as described by Jacobs (8). The type of standard and respiratory flask used with this method is of slight importance. Those devised by Dr. Bodine and used in these experiments were made of Nonsol or Pyrex glass.

Since normally no movements occur in eggs and pupæ when properly handled, it was only basal metabolism that was recorded. In all metabolism experiments during embryonic development constant temperature conditions were maintained by using Krogh's manometer immersed in a constant temperature water bath. During the course of any individual experiment temperatures were always maintained constant. The range of constant temperatures maintained during the course of the entire experiments was 21–24°C.

In following out the respiratory exchange during embryonic development, eggs known to have been recently deposited were selected and their weights obtained. Daily determinations of their CO_2 output up to the time of hatching were recorded. In following the metabolism during metamorphosis many single pupæ were used and the same procedure followed as for the eggs, until the pupæ emerged as adults.

The oxygen intake whether of eggs or pupæ during development was recorded by means of the Krogh manometer. This apparatus may also be used in making CO_2 determinations as described by Krogh (10). It has many advantages in its favor over the apparatus described by Jacobs for CO_2 determinations, some of the outstanding features being the ease with which readings are made both of oxygen intake and CO_2 output during the entire period of development of eggs or pupæ. Small objects, such as eggs or small pupæ, may be left in the manometer during their entire period of development.

As a basis for comparison of the rates of metabolism of individual pupa or eggs, the data recorded in this paper were calculated to gm. CO_2 per gm. per hour and in cu. mm. of oxygen per gm. of organism per hour.

Embryonic Development.

During embryonic development an increase of living protoplasm occurs, followed by a continuous growth of tissues and organs. According to Weismann, in holometabolous insects, at the time the larval structures develop, imaginal buds originate as separate minute cellular masses. Such imaginal buds exist for each part of the future insect. Although Kulagin (11) states that the amount of chromatin in these cells is double that contained in the definitive cells from which they arose, the imaginal buds cannot be regarded as embryonic resting stages. During larval life their growth and development, however, is greatly retarded. The processes of metabolism that take place during embryonic development as observed in the following experiments are suggestive of such conditions.

First, a formative period occurs, during which metabolic activity seems to be very low. In all embryonic development, with a few exceptions to be noted later, this formative stage is very short. As

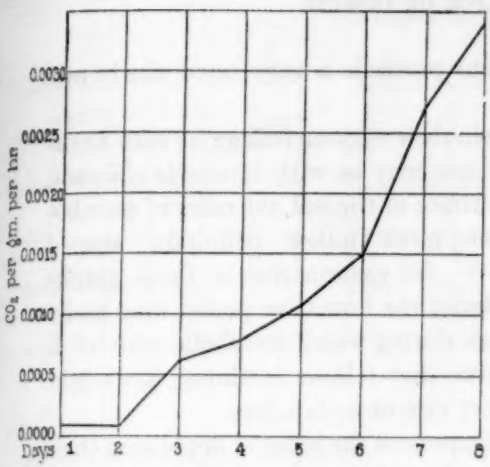
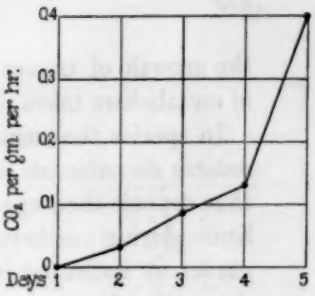


FIG. 1.



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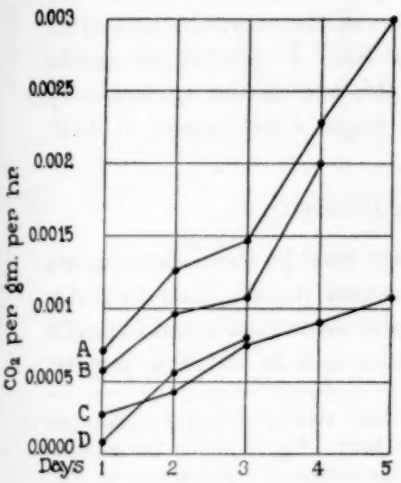

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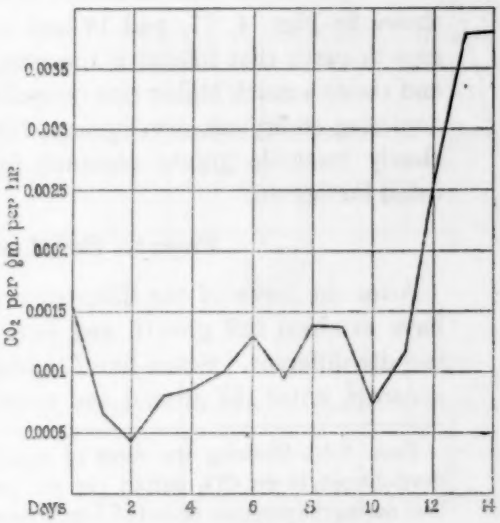


FIG. 4.

FIGS. 1–4. Showing gm. CO_2 output per gm. of organism per hour, during embryonic development. The brief formative period is indicated in Fig. 1, *Crioceris asparagi;* Fig. 2, *Hylemyia cilicrura;* and in Fig. 3, *Leptinotarsa decemlineata* by the low CO_2 output during the 1st day of development. This is followed by a marked high rate of CO_2 output until hatching as is shown by the almost straight line curves. Fig. 4 shows the curve obtained during embryonic development of *Popillia japonica*, and indicates the extended formative period lasting for over 6 days.

the growth of tissues and organs proceeds a very rapid rise in rate of metabolism takes place.

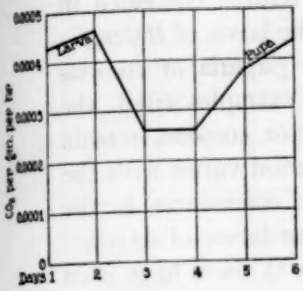
In species that usually deposit their eggs on foliage, as with *Leptinotarsa decemlineata*, or, as the case may be with *Hylemyia cilicrura*, that deposit their eggs on the surface of the soil, the rates of metabolism during embryonic development follow definitely shaped curves as indicated in Figs. 1–3. An examination of these graphs shows that in nearly every instance the formative period may easily be recognized by the short stage during which metabolic activity is at its lowest. The growth curve that follows is almost a straight line, and indicates a much higher rate of metabolism.

Other species, as *Cotinis nitida*, possess the habit of depositing their eggs in the soil, usually at some depth below the surface. The formative period in such instances seems to be greatly extended, as shown in Figs. 4, 17, and 18 and may last for many days. The growth curve that follows is the same as in the previous cases cited and shows a much higher rate of metabolism. In general, the graphs depicting embryonic development for this and similar species, very closely resemble graphs obtained for pupal development as indicated further on.

Prepupal Period and Histolysis.

After the larvæ of the different orders used in these experiments have attained full growth and have ceased feeding their behavior is quite different. Some, like *Leptinotarsa decemlineata* and *Crioceris asparagi*, enter the ground and construct cells in which to pupate.

FIGS. 5–10. Showing the rates of metabolism during prepupal and pupal development, in gm. CO_2 output per gm. per hour. Fig. 5 shows the metabolism during the prepupal period of *Leptinotarsa decemlineata*. The intense histolysis occurring during this period is indicated by the marked drop in CO_2 output lasting for several days. Figs. 6 and 7 show the CO_2 output and oxygen intake during both prepupal and pupal development of *Hippodamia convergens*. Fig. 8, *Hylemyia cilicrura* and Fig. 9, *Ancylis comptana*, also indicate prepupal and pupal development. The first few days in each of these curves represent histolysis, followed by a general rise in the curves indicating pupal development. Fig. 10, *Popillia japonica*, shows histolysis during early pupal development followed by growth. Sex differences in the rate of metabolism are also very marked, males showing a higher rate.



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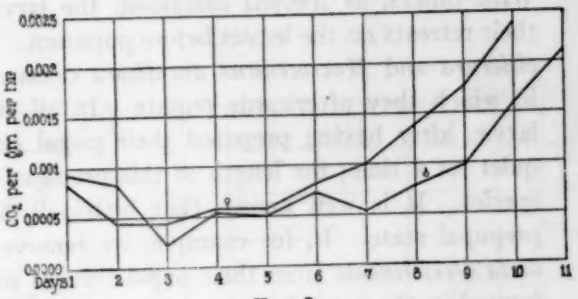

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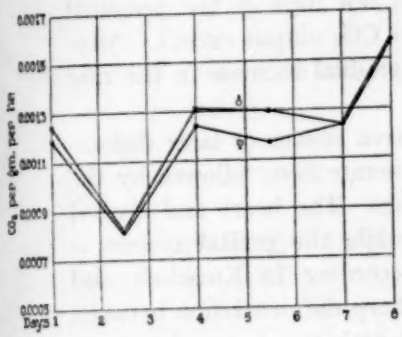

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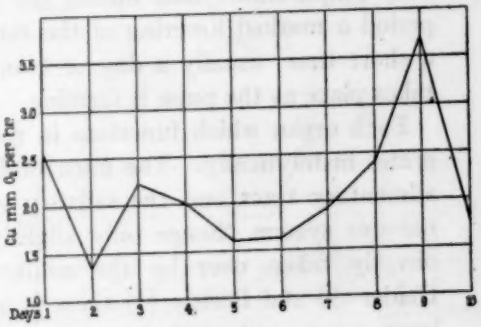

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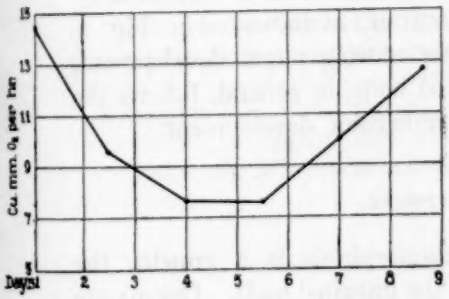

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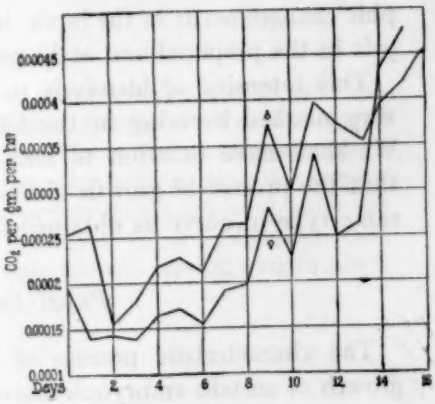


FIG. 10.

With others, as *Ancylis comptana*, the larvæ remain concealed in their retreats on the leaves before pupation. The larvæ of *Hylemyia cilicrura* and *Macrocentrus ancylivora* construct puparia or cocoons in which they afterwards pupate. In all the examples cited, the larvæ, after having prepared their pupal cells or cocoons, remain quiet for a time; the length of this prepupal period varies with the species. It is well known that histolysis first commences in the prepupal state. If, for example, we remove the larvæ of *Leptinotarsa decemlineata* from their pupal cells as soon as these have been formed in the ground, and subject them to metabolism experiments during the prepupal period, we obtain a curve as shown in Fig. 5. This graph shows that during the first few days of the prepupal period a marked lowering of the rate of CO_2 output occurs. After a short time, usually a day or two, a gradual increase in the rate takes place as the pupa is forming.

Each organ which functions in the larva sooner or later disintegrates histolytically. The muscles degenerate first, followed by the alimentary tract and the salivary glands. The heart and central nervous system change only slightly, while the genital system is directly taken over by the adult. According to Korschelt and Heider (9) and Berlese (2) there is no sharp differentiation between larva, pupa, and adult, because parallel with the destruction is an upbuilding of organs. Although we note the abrupt changes that occur outwardly, the inner development is continuous. Metamorphic changes occur in the larva, but not so intensely as during histolysis in the prepupal and early pupal state.

This intensity of histolysis in prepupal larvæ is accompanied by very marked lowering in the CO_2 output, as indicated in Fig. 5. We shall have occasion to note later, during pupal development, that the process of growth of imaginal buds, in general, follows the same type of curve as obtained for embryonic development.

Pupal Development.

The characteristic process of metamorphosis is a growth, the growth of certain embryonic tissues, the imaginal buds. Organs are not built anew, but grow from the imaginal buds which have remained

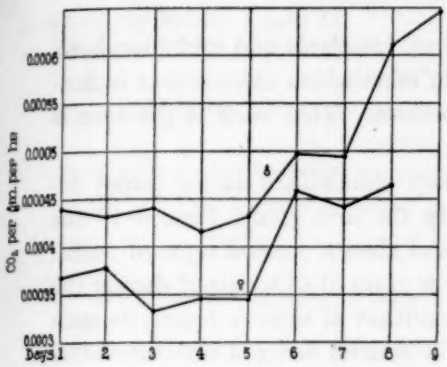
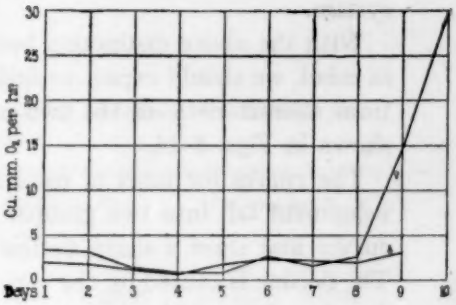


FIG. 11.



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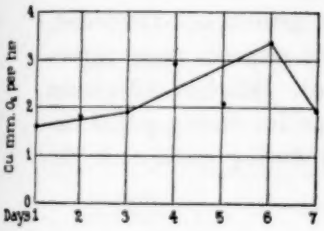


FIG. 12.

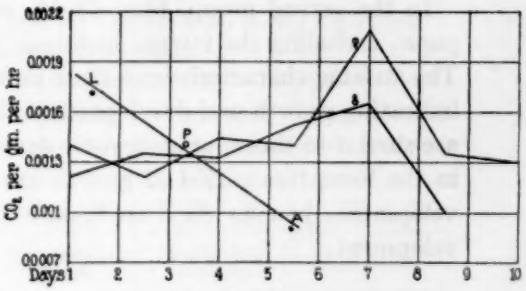


FIG. 14.

FIGS. 11–14. Show metabolism during pupal development in gm. CO_2 output per gm. per hour. Fig. 11, *Leptinotarsa decemlineata;* Fig. 12, *Crioceris asparagi;* Fig. 13, *Macrocentrus ancylivora;* and Fig. 14, *Ancylis comptana.* Note the absence of the intense histolysis that takes place in the prepupal development as compared to Figs. 5–10. The above curves show growth and development and are comparable to embryonic development (see Figs. 1–4). A sex difference is also evident, especially in *Leptinotarsa decemlineata*, the male showing a higher rate of metabolism.

latent since their embryonic origin. A large growth of certain organs does occur, as for example in wings and appendages. Parallel with this growth notable changes occur in the heart and nervous system.

With the above distinction between histolysis and metamorphosis in mind, we should expect to find in metabolism experiments indications characteristic of the two processes. That such is the case is shown in Figs. 5–14.

The curves for rates of respiratory metabolism during pupal development fall into two groups. In the first group, Figs. 5–10 the curves first show a sharp decline and then a general upward trend. The decline is caused by the very low rates of CO_2 output during the first few days of histolysis, a concomitant of intense histolysis such as occurs in the prepupal state. The general rising of the curve after this initial low phase of metabolism accompanies metamorphosis, the growth of imaginal buds.

In the second group, Figs. 11–14, are records obtained from the pupæ, excluding the intense histolytic process of the prepupal state. The striking characteristic of these curves is a general upward trend, indicating growth and development. In many respects these curves are similar to those for embryonic development. Marked differences in the formative period of growth exist in species during pupal development, just as they are found to exist during embryonic development.

The Energy Required in Embryonic and Metamorphic Development.

During the embryonic and metamorphic development, fat seems to be largely burned. The oxidation of fat must generate energy for development. The chemical processes concerned in the utilization of fat are not definitely known at present. According to Bayliss (1) and Mathews (12) the fats are first hydrolyzed before oxidizing, the fatty acids are then probably united to some substance which hastens their oxidation. The result of this oxidation is the liberation of a large amount of energy, carbon dioxide, and water. Since the egg contains a relatively larger amount of fat than does the pupa, we may expect the generation of energy to be greater during embryonic development.

To show the relative amounts of energy required during embryonic and pupal development, the total CO_2 output or oxygen intake per gm. per hour was calculated as averages; the results tabulated are shown in Tables I and II.

TABLE I.

The Total CO_2 Output per Gm. per Hour during Embryonic and Metamorphic Development.

Leptinotarsa decemlineata.		*Hylemyia cilicrura.*		*Popillia japonica.*		*Epilachna borealis.*	
Egg.	Pupa.	Egg.	Pupa.	Egg.	Pupa.	Egg.	Pupa.
0.00489 ± 0.00011	0.00343 ± 0.00015	0.0123 ± 0.0018	0.00789 ± 0.0001	0.0189 ± 0.0014	0.00387 ± 0.0002	0.0110 ± 0.0006	0.0051 ± 0.0007

TABLE II.

The Total Oxygen Intake in Cu. Mm. per Gm. per Hour during Embryonic and Metamorphic Development.

Leptinotarsa decemlineata.	*Crioceris asparagi.*	*Popillia japonica.*	*Cotinis nitida.*	*Epilachna borealis.*
Egg.	Egg.	Egg.	Egg.	Egg.
2,832 ± 62	2,416 ± 49	2,705 ± 60	2,229 ± 47	1,175 ± 30
Leptinotarsa decemlineata.	*Crioceris asparagi.*	*Hippodamia convergens.*	*Macrocentrus ancylivora.*	*Ancylis comptana.*
Pupa.	Pupa.	Pupa.	Pupa.	Pupa.
1,270 ± 94	1,902 ± 35	1,099 ± 80	2,222 ± 21	3,572 ± 33

The tables indicate quite clearly that there are marked differences in metabolic rates between egg and pupa. Indeed, so great is this difference that with some species the rate during embryonic development is nearly treble that during metamorphosis. In some species egg incubation requires a much shorter time than pupal development. These results, show, therefore, an actually higher rate of metabolism for embryonic development than for metamorphosis. Tangl (13), it is interesting to note, compared the rates of embryonic development of *Bombyx mori* and the chick embryo and found the rates to be comparable.

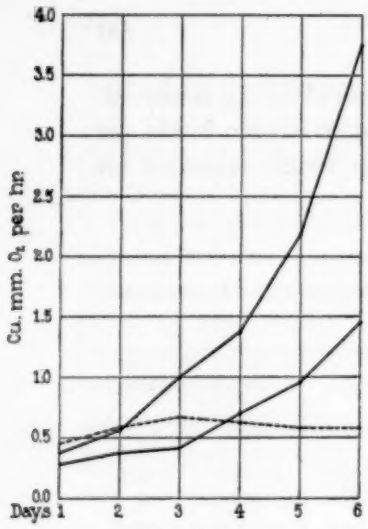
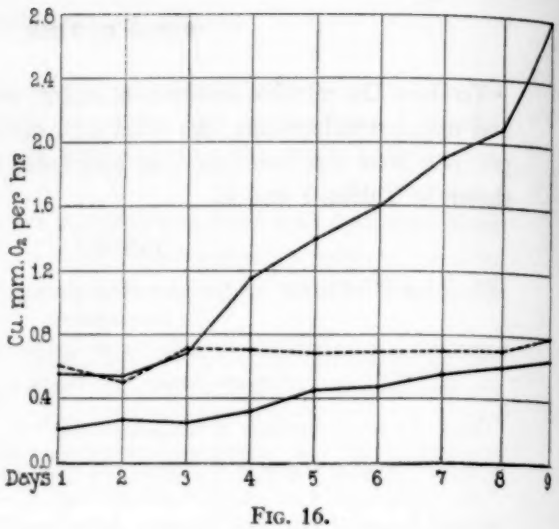


FIG. 15.



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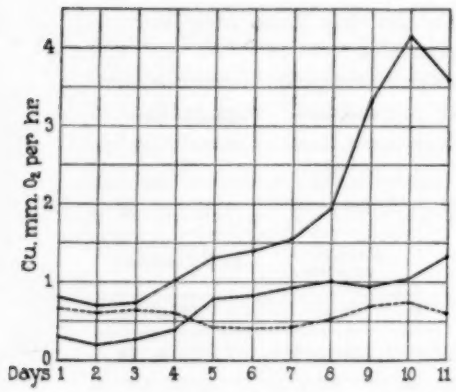

FIG. 17.

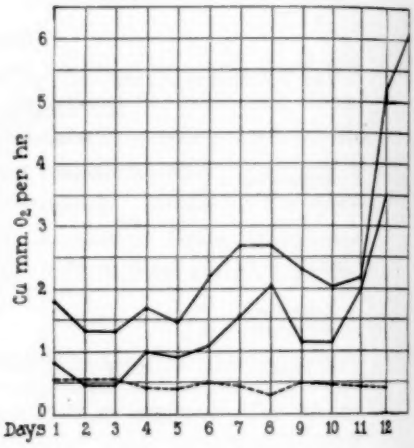

FIG. 18.

FIGS. 15–18. Showing the oxygen intake, the CO_2 output, and respiratory quotient during embryonic development. Fig. 15, *Leptinotarsa decemlineata;* Fig. 16, *Anasa tristis;* Fig. 17, *Popillia japonica;* Fig. 18, *Cotinis nitida.* The upper solid line curve in each figure represents the oxygen intake, the lower solid line curve the CO_2 output. The dotted line curve shows the respiratory quotient. Note the brief formative period as shown in Figs. 15 and 16, as compared to the more extended formative stage in Figs. 17 and 18. The former species deposit their eggs on foliage, the latter in the soil.

Respiratory Quotient.

Through the investigation of many workers it has been experimentally demonstrated that a low respiratory quotient indicates that the substances catabolized are chiefly fat and protein, and that a high quotient indicates the substances to be chiefly carbohydrates and protein.

Both Tangl and Weinland have obtained low respiratory quotients during metamorphosis of pupæ, the daily quotients obtained by Weinland varying from 0.46 to 0.76. Similar strikingly low respiratory quotients were obtained by the author with daily variations ranging from 0.42 to 0.71, as shown in Figs. 15–18.

The embryonic development of *Anasa tristis* shows (Fig. 16) an initial respiratory quotient of 0.521, rising on the 3rd day to 0.732; the latter is practically maintained thereafter throughout development. In Fig. 15 referring to the embryonic development of *Leptinotarsa decemlineata* we have a similar condition, with a quotient ranging daily from 0.512 to 0.68. *Cotinis nitida* gives a much lower quotient during embryonic development, seldom rising above 0.524 and for several days even dropping to 0.413. A similar low respiratory quotient is found for *Popillia japonica*, but here it rises during the last few days of development to 0.732 (Figs. 17 and 18).

Many investigators have obtained low respiratory quotients with hibernating mammals such as the bat and marmot. Similar low quotients ranging from 0.41 to 0.71 have also been obtained by the author (unpublished data) for hibernating insects, while the active forms of the same species gave quotients ranging from 0.72 to 0.8. No satisfactory explanation has as yet been brought forward to explain the occurrence of a quotient lower than 0.7, although it is surmised that the substances oxidized are chiefly fat. Thus Bohr and Hasselbalch (4) believe that the variation in the quotient observed with the chick embryo is due to the decomposition of fat, not at one time, but in successive stages. This agrees with Bayliss, who holds that in the oxidation of fats there first occurs a splitting into fatty acids, followed by a quick oxidation of the lower fatty acids. Weinland is of the opinion that perhaps the oxygen intake is in some way held back in the organism. Bayliss also states that it can be shown by the increase of weight that oxygen is actually retained.

The Reaction of Body Fluids.

The pronounced changes in CO_2 output found during early and late embryonic development, prepupal and pupal changes, led to the assumption that perhaps changes in the pH of the body fluids were also taking place. It is not definitely proved as yet that we can correlate a low CO_2 output with an acid condition and, conversely, a high rate of CO_2 output with a neutral or slightly alkaline medium, for low rates of CO_2 output do not always seem to be associated with an acid medium. Bishop (3), however, is of the opinion that a change in pH and total acid content at the beginning of pupation in bee larvæ produces a condition favorable to autolysis.

Experiments were made to test the reaction of the fluids of larvæ, pupæ, and eggs of the species recorded in this paper. The results obtained show marked changes in pH before pupation and during the last stages of pupal development. Owing to the extremely small amounts of fluid available in the material studied (not more than a drop or two can be obtained) the colorimetric spot-plate method was adopted. This method was found to give approximate results. The procedure was as follows: Drops of fluid obtained from the insect were diluted with a drop of pure water in a depression on the spot-plate and compared to equal size drops of standard buffer solutions, using one or more indicators to check a given result. When two indicators gave approximately the same pH, it was concluded to be the pH of the fluid in question. As indicators brom-thymol blue, brom-cresol purple, and phenol red were used. Determinations could be made in less than 10 seconds. Every precaution was used to insure uniform determinations. Any change in the reaction of the fluid must, therefore, be attributed to an actual change in its acidity or alkalinity.

In Table III the results are tabulated according to species. They reveal in some cases pronounced changes from a neutral to an acid reaction of the fluids at the termination of the larval period and in the early pupal stage. The acid condition, moreover, prevails throughout at least the first half of pupal development. Afterwards, usually at about the time the adults are ready to emerge, the pH changes again toward a neutral and, in some cases, to a slightly alkaline reaction.

As for changes from an acid to a neutral reaction during embryonic development, positive results could be obtained only in a few species, such as *Cotinis nitida*, *Hylemyia cilicrura*, and *Anasa tristis*. Other species, as *Leptinotarsa decemlineata*, showed very slight variations or none at all. Throughout all the stages of *Epilachna borealis*, a very marked acid reaction prevails. During the growth of *Anasa tristis* a very gradual change from an acid to neutral state occurs. In this species metamorphosis is incomplete.

All the species having complete metamorphosis except *Epilachna borealis* show distinct changes in pH—a change from neutral to acid reaction just previous to pupation, and from acid to neutral reaction as metamorphosis nears completion.

TABLE III.

The Reaction of Body Fluids.

Species.	Egg.		Larva.		Pupa.	
	Early.	Late.	Early.	Late.	Early.	Late.
	pH	*pH*	*pH*	*pH*	*pH*	*pH*
Leptinotarsa decemlineata	6.8	6.8	6.8	5.9	5.9	6.8
Colinis nitida	6.2	7.1	7.3	—	—	—
Popillia japonica	7.1	7.1	—	—	—	—
Ancylis comptana	—	—	6.8	5.9	5.9	6.8
Epilachna borealis	5.9	5.9	5.9	5.9	5.9	5.9
Hylemyia cilicrura	5.9	6.6	7.1	6.6	5.9	6.6
Anasa tristis	6.2	6.4	6.6	6.6	6.8	6.8
Pieris rapæ	—	—	6.4	6.2	5.9	6.4

The destruction of larval tissues during histolysis has been described by many workers as due to phagocytes. Others, like Berlese, discard this theory entirely. Breed (5) found no evidence of phagocytes in the degeneration of larval muscle tissues of Coleoptera, and believes that histolysis is entirely a chemical process. Herbst (7) found that if sea water is deprived of its calcium, the blastomeres of sea urchin eggs fall apart; whereas if calcium is added, the cells cohere again. Bishop (3) is of the opinion that a change in metabolic equilibrium is the immediate cause of tissue autolysis, a more acid reaction acting as a contributing cause to the acceleration of the process. Whether an acid reaction is essential for histolysis is not definitely known.

SUMMARY.

1. For species of insects that deposit their eggs on foliage, as *Leptinotarsa decemlineata*, *Crioceris asparagi*, and *Anasa tristis*, the rates of metabolism as measured by the CO_2 output or the oxygen intake indicate, during embryonic development, a short formative period, followed by a very active extended growth. Those species that deposit their eggs in the soil, as *Cotinis nitida* and *Popillia japonica*, show that the formative period is greatly extended and the growth period similarly lengthened.

2. It has been shown from metabolism experiments that intensive histolysis occurs during the prepupal period and becomes less intensive during pupal development.

3. Metabolism experiments show a greater amount of energy change during embryonic development as compared to the energy developed during metamorphosis. This is shown by the greater CO_2 output and by the oxygen intake.

4. Low respiratory quotients, varying from 0.42 to 0.71, have been obtained during the embryonic and pupal development of insects, resembling similar low quotients obtained with hibernating forms.

5. Changes from a neutral to an acid reaction (pH 6.8 to 5.9) have been observed to take place in some species during prepupal and early pupal development. As metamorphosis is completed a converse change occurs (pH 5.9 to 6.8). Changes in pH from an acid to neutral reaction were also observed to take place in some species, as *Cotinis nitida* and *Hylemyia cilicrura*, during embryonic development.

The author desires to acknowledge his appreciation for the facilities extended him by the Department of Zoology of the University of Pennsylvania during these investigations; and particularly is he under deep obligation to Dr. J. H. Bodine for many helpful suggestions during the progress of the work.

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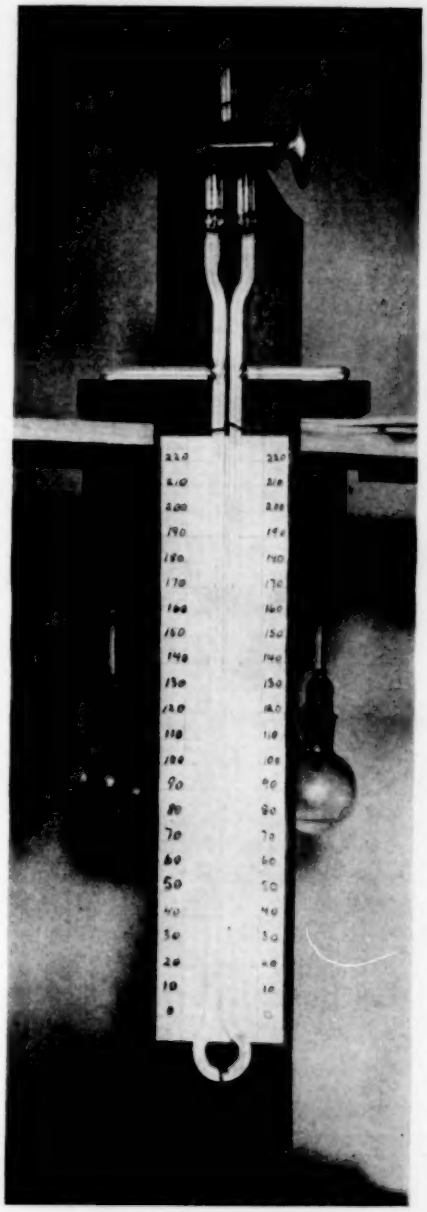
EXPLANATION OF PLATE 4.

FIG. 1. Krogh's manometer, the apparatus used for determining the respiratory gas exchanges of insects during embryonic and metamorphic development.



(Fink: Metabolism of insects.)

NEUROID TRANSMISSION IN CILIATED EPITHELIUM.

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I.

INTRODUCTION.

A study of cilia raises the question to what extent their activity is determined directly by the physicochemical conditions of the environment, and to what extent it is in physiological correlation with other activities of the organism. There is undoubted evidence that certain cilia act in relative independence of the rest of the animal to which they belong. Such cilia are indifferent to removal of the ciliated tissue, but show great susceptibility to changes of the environment.

Gray (1920, *a;* 1920, *b*) in two recent papers, has given an account of cilia on the gills of *Mytilus*. Cilia on excised gills continue undisturbed beating for several days when kept in sea water. Yet with change of the pH value of the sea water from 5.5 to 6.5 the cilia show an immediate suspension of beating. If this critical pH value is not overstepped, restoration of hydroxyl ions is followed by resumption of activity. If on the other hand the pH is carried above 9, the epithelium breaks up, though the cilia continue to beat. Furthermore, the presence of sodium, potassium, calcium, and magnesium in certain defined amounts is necessary for normal beating. Von Brücke (1916) demonstrated in a striking way the independence and autonomy of cilia of the epithelium of the frog's mouth. A piece of epithelium was removed, turned through ninety degrees, and replaced. The tissue so treated continued to live and its cilia to beat normally. They did not, however, orient themselves to their new position, but continued their old line of beat, at right angles to the surrounding cilia.

There is at the same time a coordination of all the cilia in the same row, whereby each cilium is slightly in advance in phase of the cilium next below it in the line of beat, regardless of the rate of the cilia. This condition has been studied by Kraft (1890) in the ciliated epithelium of the pharynx of the frog. In his observations he determined the rate of cilia by microscopic inspection, carrying out his experiments on the stage of the microscope. He confirmed Grützner's observation that

injury to *Oberzellen* (*i.e.* cells relatively high up in a row of beating cilia) caused inhibition of *Unterzellen* (cells below them in the line of beat), as well as the local inhibition. On the other hand he failed to find any inhibition of *Oberzellen* following injury to *Unterzellen*. He also substantiated König's work showing that facilitation of *Oberzellen*, produced by gentle stroking with a fine brush, was followed by increase in rate of beat of the *Unterzellen*, though similar stimulation of *Unterzellen* had a much more delayed and less marked effect on *Oberzellen*. Dropping a fragment of cork on the tissue likewise produced both local stimulation and stimulation of *Unterzellen*, though *Oberzellen* were scarcely affected.

In all these experiments cilia to one side of the row in question were uninfluenced. To answer the question whether this effect of *Oberzellen* on *Unterzellen* is merely mechanical or represents true physiological coordination, Kraft made use of a small box, 30 mm. by 10 mm. by 15 mm., with a glass bottom, brass sides, and a top of thin cover-glass. By two glass partitions at right angles to the longer side and to the bottom, the box was divided into three chambers, the middle one 5 mm. wide. The whole box could be placed on the stage of the microscope and water of desired temperatures kept circulating through the three chambers. A piece of epithelium could be then stretched over the cover-glass and the behavior of the cilia observed microscopically under different conditions. In one set of experiments the epithelium was arranged with its rows of cilia at right angles to the partitions, and water at 0°C. was passed through the middle chamber while water at 10–12° circulated through the end chambers. Under these conditions cilia over the end chambers maintained a slow beating; those over the middle chamber were quiescent. If now cilia over the upper end chamber (with reference to the line of beat) were stimulated by gentle stroking with a brush, cilia over the lower end chamber showed a corresponding increase of activity, though cilia over the middle chamber continued inactive as before. If a stimulating temperature of 15° in the upper chamber was substituted for the mechanical stimulus the results were the same except that in this case the cilia over the middle chamber also became active, though more slowly, and they themselves in turn produced further stimulation of the cilia over the lower chamber. In another set of experiments water of 0°C. was passed through both end chambers and water of 20°C. through the middle chamber. In this case the cilia over the lower chamber showed a marked increase of beat under the influence of the warm stimulus applied to the cilia above them, but cilia over the upper chamber showed only a very slight increase. As a result of these experiments Kraft concluded: (1) that cilia are not subject to the control of the nervous system; (2) that there is an inner, physiological conduction of the effects of a stimulus from *Oberzellen* to *Unterzellen* but not in the reverse direction nor laterally; and (3) that there is a slight mechanical effect on adjacent cilia produced by cilia under stimulation. He distinguishes "eine aüssere, mechanische Leitung" and "eine innere, nervenartige Leitung der Eregung."

Engelmann (1898) in his article on cilia recognizes this physiological interconnection between ciliated cells, though he does not regard it as thus limited in direction. He says, "Il est évident que ce phénomène doit être rapproché de la conduction dans le coeur et dans les muscles lisses, où la conduction de l'excitant physiologique se fait par la propagation d'une action moléculaire entre cellules excitables, placées au contact les unes des autres."

Other evidence recently brought forward indicates that in some animals there is a control of cilia by the nervous system. Göthlin (1920), working on *Beroe* demonstrated that by use of mild electrical currents, 1 to 2 ma./ sq. cm., inhibition of the swimming plates could be produced without contraction of the meridional rows. The same effect was produced by gentle mechanical stimulation of the edges of the mouth and of the polar fields, and was observed even after removal of the sense body at the aboral end. Anesthesia with chloral hydrate, which did not interfere with the beating of the swimming plates, prevented this inhibition. Moreover swimming plates on fragments of epithelium separated from the underlying tissue showed no response to the inhibitory stimuli, though in cases where the underlying tissue was present inhibition was observed. From this Göthlin concluded that the swimming plates are under control of a nervous mechanism, and that the stimulus, acting directly on epithelial sense organs, affects the swimming plates only indirectly, by way of this nervous mechanism. Anesthesia or isolation, eliminating nervous processes, renders the swimming plates indifferent to the stimulus.

Copeland (1919, 1922) maintains that there is nervous control of cilia on the feet of certain mollusks. His observations are on *Alectrion trivittata*, *Alectrion obsoleta*, *Polinices heros*, and *Polinices duplicata*. He shows that the pedal cilia of these snails are associated with locomotion, and beat in coordination over the whole foot. In *Alectrion* stimulation of one of the pedal tentacles produces immediate activity of all the cilia on the foot. In *Polinices* the pedal cilia all become active simultaneously as if excited by an outside (nervous) impulse. In this animal cilia over an area isolated by cutting from adjacent cilia show no alteration in beat if their connections with the underlying tissue are not disturbed. On the other hand, isolation of such an area from the subjacent tissue, connection with the surrounding tissue being left intact, produces loss of normal activity. Copeland regards these facts as indicating nervous control of the pedal cilia of these snails.

Thus there are three groups of experiments which emphasize respectively (1) relative independence of ciliary activity of other organic processes, (2) interconnection of different parts of the same ciliated epithelium, and (3) relations of ciliary activity to the rest of the organism, in particular through the nervous system.

II.

Method and Apparatus.

In the following experiments a study was made of the correlation in rate of beat of the cilia on the gill of *Unio*, and of the supposed transmission by means of which such correlation is maintained, a transmission which Kraft described as "eine innere, nervenartige Leitung" and which together with some other types of primitive conduction has been more recently designated "neuroid transmission" (Parker, 1910). These cilia cover both surfaces of both inner and outer gills, serving to drive water through the gills and to convey the extracted food particles. Those on the outer gill beat upward, carrying the particles to the upper edge of that gill, where they are received by other, specialized cilia and swept forward in a groove-like depression to the mouth region, there to be received by the palps. Those on the inner gill beat downward, driving the food fragments to the lower margin of that gill, where they suffer a similar fate. In the following work only the inner gills were used since these were larger and since in the case of females, which predominated in the animals brought to the laboratory, the outer gills were filled with eggs. The gill to be examined was removed from the animal and allowed to remain half an hour or more in tap water until the excess mucus secreted in consequence of cutting was swept off and until the possible mechanical effects of handling might have subsided. It was then tested for conduction of the stimulating effects of different temperatures with the apparatus and in the manner to be described.

A rectangular depression, of length and width corresponding approximately with the dimensions of the larger gills, was hollowed out of a block of wood, lined with wax, and divided into two chambers by a slate partition at right angles to the longer sides (Fig. 1). Through the opposite walls of each chamber opened an inlet and an outlet formed of tight fitting glass tubing. The chambers were sealed on top either with a large cover-glass cemented down with a mixture of bees-wax and rosin or with a thin sheet of mica fastened down with shellac. This latter arrangement was found to be more satisfactory and was used in the later experiments. The chambers were thus rendered water-tight save for outlets and inlets. On the

outside around the edges of the cover-glass or sheet of mica a low bulwark of wax was erected so that the upper surface of the glass or mica might be flooded with a thin layer of water.

During an experiment, water of desired temperatures was syphoned through the chambers and any trapped air removed at the start by tipping the apparatus. The gill was laid in the desired position over the chambers and covered with a thin sheet of water to facilitate the

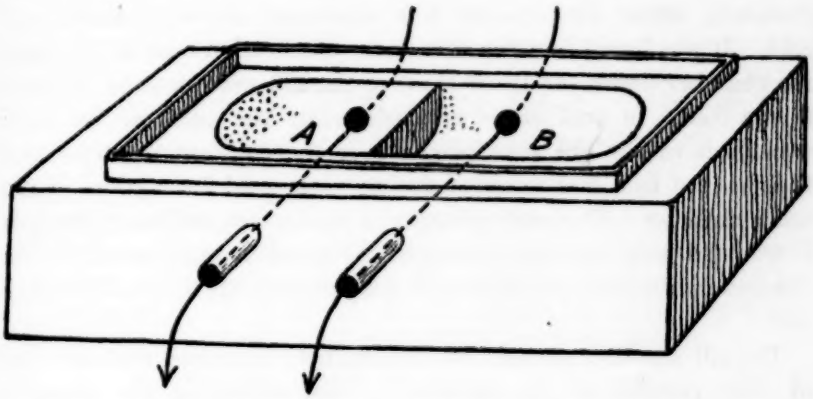

FIG. 1. A wooden block partly hollowed out above and arranged to apply temperature changes to the gill of *Unio*. The two chambers, *A* and *B*, are separated by a narrow partition and sealed above by a thin sheet of mica or by a cover-glass. The piece of gill was laid on this mica or cover-glass and flooded with a small amount of water which was retained by the surrounding low barrier. The temperatures of the two chambers were maintained by currents of water led through them as indicated by the arrows. These temperatures affected the gill which was placed on the mica or cover-glass immediately above the two chambers and partly over each.

action of the cilia. A light fragment of a mm. rule was held over the surface of the gill, parallel to the rows of cilia, and grains of carmine were dropped near it on the gill. The time required for a particle to travel 1 cm. under the influence of the cilia was recorded with a stop-watch. An average of several such times was taken as representing the rate of the cilia under the given condition. Since the syphons could be changed without emptying the chambers the whole arrangement was left nearly undisturbed throughout the experiment.

III.

EXPERIMENTAL RESULTS.

In the first set of experiments an excised gill was laid on the coverglass, overlapping both chambers and with its rows of cilia at right angles to the partition. The *upper* two-thirds of the gill, on which the rate of ciliary beat was observed, overlay the chamber kept at a constant low temperature. The lower third overlay the other chamber, whose temperature was alternated between warm and cold. It was found that the cilia under observation beat faster when the chamber under the lower third of the gill was warm than when it was cold. In another set of experiments the cilia on the *lower* two-thirds of the gill were placed over the constant chamber and their rate of beat was recorded during warm–cold fluctuations of the other chamber. The same changes in rate corresponding to changes in temperatures was observed as in the previous experiments. The changes in the observed rates and temperatures are shown in Figs. 2 and 3.

The gill was then placed, overlapping both chambers with its rows of cilia, parallel to the partition. Cilia overlaying the chamber that was kept at a constant low temperature were then observed while the temperature of the other chamber was varied between cold and warm. The distance of the rows of observed cilia from the partition was between 4 and 8 mm. The same fluctuations in rate corresponding to changes in temperature of the variable chamber were observed. The results are shown in Fig. 4. As in the two previous cases a study was made of the maximum distance from the partition at which this effect was observable. It was found that the fluctuations did not occur beyond 9 to 11 mm. from the partition. A typical set of three readings taken at points within 11 mm. of the partition is given in Fig. 5.

In order to check these results a gill was placed upon the coverglass as before with its rows of cilia parallel to the partition. The rate of beat of cilia over the chamber kept at constant temperature was then observed in the usual manner during several alternations of the temperature of the other chamber. If the gill showed the usual changes in rate corresponding to the alternating changes in

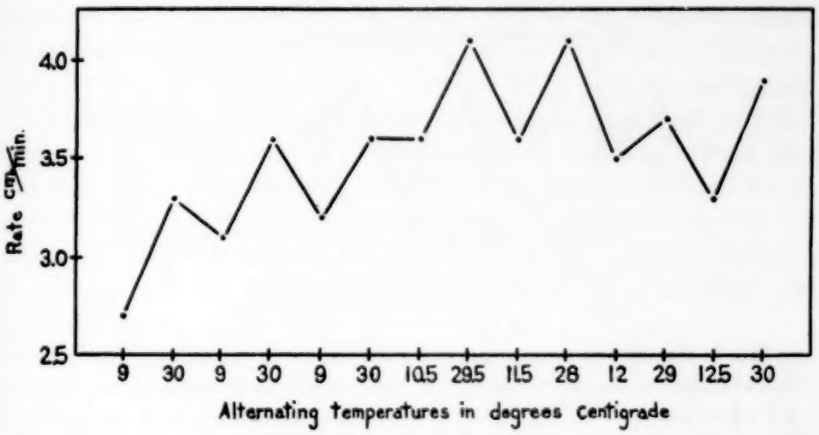

FIG. 2. Changes in average rates of movement, cm. per minute, shown by particles of carmine driven by the cilia of the gill of *Unio* in which the region of the gill under observation was kept at 10°C. and an adjacent region *lower* in the line of ciliary beat was subjected to temperatures alternately low (about 10°C.) and high (about 30°C.). In no instance did the probable error of the observations upon which each average was based exceed 3.1 per cent of such average.

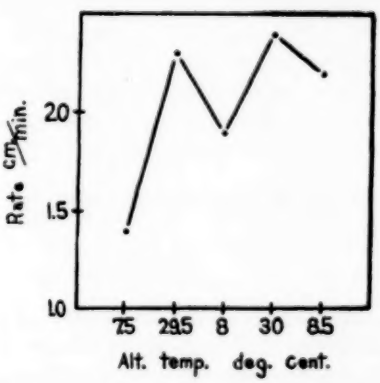

FIG. 3. Changes in average rates of movement, cm. per minute, shown by particles of carmine driven by the cilia of the gill of *Unio* in which the region of the gill under observation was kept at 7.5°C. and an adjacent region *higher* in the line of ciliary beat was subjected to temperatures alternately low (about 8°C.) and high (about 30°C.). In no instance did the probable error of the observations upon which each average was based exceed 2.7 per cent of such average.

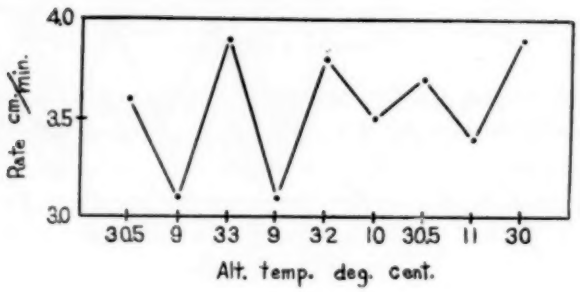

FIG. 4. Changes in average rates of movement, cm. per minute, shown by particles of carmine driven by the cilia of the gill of *Unio* in which the region of the gill under observation was kept at 8°C. and an adjacent region *to one side* of the line of ciliary beat was subjected to temperatures alternately low (about 10°C.) and high (about 30°C.). The near edge of the observed region was from 5 to 8 mm. distant from that of the other region. In no instance did the probable error of the observations upon which each average was based exceed 4.6 per cent of such averages.

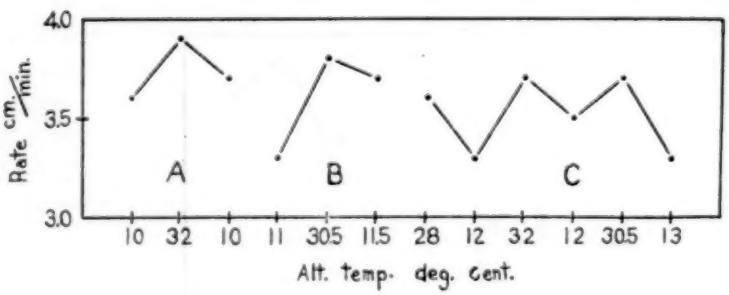

FIG. 5. Changes in average rates of movement, cm. per minute, shown by particles of carmine driven by the cilia of the gill of *Unio* in which the region of the gill under observation was kept at from 10–12°C. and an adjacent region *to one side* of the line of ciliary beat was subjected to temperatures alternately low (about 11°C.) and high (about 30°C.). The near edge of the observed region was distant from that of the other region in one set of observations (*A*) 20 to 22 mm., in a second (*B*) 14 to 16 mm., and in third (*C*) 9 to 11 mm. In no instance did the probable error of the observations upon which each average was based exceed 2 per cent of such averages.

temperature it was regarded as normal. Thereupon, a cut was made completely across the gill, parallel to the partition and a little to one side of it over the constant chamber so as to sever all organic connection between the region of alternating temperatures and the cilia under observation. The alternations of temperature on the variable side were continued, and the readings were made as usual on the same cilia as before. Under these conditions there was no longer any significant change in ciliary rate corresponding to changes in temperature of the variable chamber. Typical readings are given in Fig. 6.

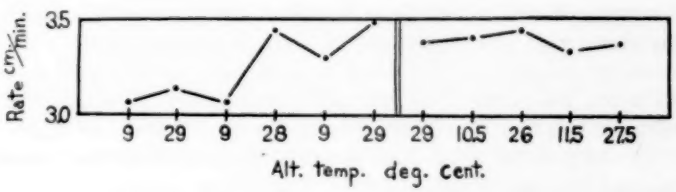

FIG. 6. Changes in average rates of movement, cm. per minute, shown by particles of carmine driven by the cilia of the gill of *Unio* in which the region of the gill under observation was kept at 9°C. and an adjacent region *to one side* of the line of ciliary beat was subjected to temperatures alternately low (about 10°C.) and high (about 28°C.). After six observations had been made the gill was cut in two between the region of observation and the other region and five more observations were made. The cut is indicated on the graph by the vertical double line. In no instance did the probable error of the observations upon which each average was based exceed 2.3 per cent of such average.

Throughout these experiments the temperature of the constant chamber had been low, equal to the low temperature alternating with the high, on the variable side. The question remained open, therefore, whether it was the effect of the cold, or the warmth, or both that was transmitted across the gill to produce the fluctuations observed. To test this point the temperature of the constant chamber was made the same as the warm alternating temperature. In this case it was assumed that cilia directly subjected to the warm stimulus would not be further excited by the same stimulus applied at a distance and acting only indirectly, so that any alternations of the rate must be due to the effects of the cold chamber, transmitted

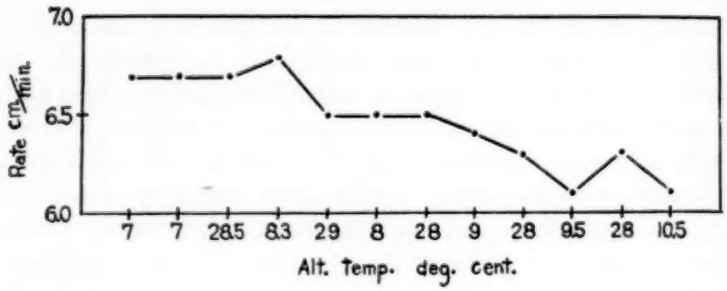

FIG. 7. Changes in average rates of movement, cm. per minute, shown by particles of carmine driven by the cilia of the gill of *Unio* in which the region of the gill under observation was kept at the relatively ***high*** temperature of 29°C. and an adjacent region *to one side* of the line of ciliary beat was subjected to temperatures alternately low (about 8°C.) and high (about 28°C.). The near edge of the observed region was from 5 to 8 mm. distant from that of the other region. In no instance did the probable error of the observations upon which each average was based exceed 3.9 per cent of such average.

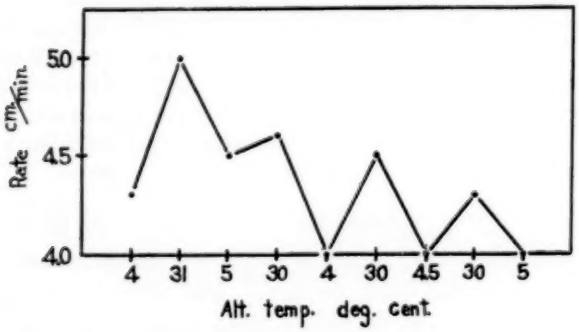

FIG. 8. Changes in average rates of movement, cm. per minute, shown by particles of carmine driven by the cilia of the gill of *Unio* in which the region of the gill under observation was kept at the ***medium*** temperature of 15°C. and an adjacent region *to one side* of the line of ciliary beat was subjected to temperatures alternately low (about 4.5°C.) and high (about 30°C.). The near edge of the observed region was from 5 to 8 mm. distant from that of the other region. In no instance did the probable error of the observations upon which each average was based exceed 3.6 per cent of such average.

through the gill. The results showed that under these conditions there was no significant fluctuation in ciliary rate corresponding with the temperature alternations of the variable side (Fig. 7). If, however, the constant temperature was midway between that of the cold and the warm the usual fluctuations were observed. Typical results are given in Fig. 8.

IV.

DISCUSSION.

It may be objected that these results are vitiated by the possibility of a spread of the warmth through the cover-glass, or gill, or both to the supposed constant area under observation. An answer to this objection was made possible by the kindness of Dr. F. G. Benedict, who offered the use of his thermopile for the measurement of the temperature gradient both on the bare cover-glass and on the gill over the region concerned in the experiment. These measurements showed a greater temperature uniformity on each side of the partition in the case of the bare cover-glass than in the case of the gill, but in either case uniformity sufficient to warrant confidence in the results. In the case of the bare cover-glass, if the temperature of the cold side at a point 8 mm. from the partition is represented by C, the gradient taken at 2 mm. intervals across the glass surface to a point 8 mm. from the partition on the warm side may be indicated as follows: *cold side*, 8 mm. C, 6 mm. C $+0.18°$, 4 mm. C $+0.11°$, 2 mm. C $+0.5°$; *over the partition*, C $+3.5°$; *warm side*, 2 mm. C $+8.0°$, 4 mm. C $+9.0°$, 6 mm. C $+9.7°$, 8 mm. C $+10°$. In the case of the gill, by using the same notation, the gradient may be expressed thus: *cold side*, 8 mm. C, 6 mm. C $-0.21°$, 4 mm. C $+0.14°$, 2 mm. C $+ 2.21°$; *over the partition*, C $+ 4.76°$; *warm side*, 2 mm. C $+ 13.6°$, 4 mm. C $+ 14.42°$, 6 mm. C $+ 15.19°$, 8 mm. C $+ 15.54°$. It is evident that points more than 4 mm. from the partition on the cold side are not appreciably affected in temperature by the warm water circulating through the opposite chamber. It was at such points that the positive results were obtained; these results are not, therefore, invalidated by a spread of heat. The uniformity of the warm side is somewhat less pronounced, but as the results here were

negative *despite* any possible spread of heat they may be safely accepted.

These results reveal the fact that the temperature difference between the two sides of the cover-glass, as well as between the two sides of the gill on the cover-glass, is considerably less than the difference between the two streams of water circulating through the two chambers, so that the effective stimulus is less than what would be supposed from an inspection of the tables. This is due to the fact that the upper surface of the cover-glass or gill is in contact with the air, a condition which tends to equalize temperature differences on the two sides.

In judging these results it should be kept in mind that the rate of the cilia, as measured by the speed of the carmine particles, shows considerable variation, probably due to interference of mucus, even when the conditions of the environment are unchanged. The fluctuation of rate of beat accompanying the regular alternation of cold and warm is thus superposed on an irregular base line and itself appears irregular, though in reality it may not be so. In addition, variations in size and shape of the particles of carmine probably introduce further irregularities. Allowance must be made for these factors.

As to the nature of the conduction little may be said except that it is an example of the type of transmission termed "neuroid." A similar condition may be observed in a finger of the sponge *Stylotella*. If a cut be made on a finger of this sponge within 1.5 cm. of the osculum, the osculum is observed to close after a brief delay, indicating the reception of an impulse spreading out from the region of injury. The sponge is, however, without nervous tissue (Parker, 1910).

The conduction recorded in these experiments differs from that investigated by Kraft (1890) in the epithelium of the frog, for it is unlimited in direction, whereas that of the frog proceeds only from *Oberzellen* to *Unterzellen*. The two are alike, however, in their relation to temperature. By both the facilitating effects of warmth are transmitted but not the inhibitory effects of cold. Kraft does not make this statement explicitly in his paper, but it is evident from his results. For when the upper of his three chambers was kept

between 12° and 15°, the middle one between 0° and 2°, and the lower one between 12° and 15°, cilia over the upper and lower chambers showed a sluggish beating and those over the middle chamber remained nearly quiescent. But when the temperature of the upper chamber was raised to 20°, cilia over both upper and lower chambers showed rapid increase of beating. Here cilia over the lower chamber were influenced by two stimuli applied above them, one cold and one warm, each differing by the same number of degrees from the temperature of the lower chamber, the cold, however, being nearer than the warmth. Nevertheless the warmth is the effective one. Moreover, at the beginning of the experiment cilia over upper and lower chambers have the same rate of beat, though the cold of the central chamber can be transmitted only to the *Unterzellen*, if it is transmitted at all, according to Kraft's results. Hence Kraft's results also point to the transmission of the effects of warmth but not of cold.

In this connection it may be noted that in the case of the thermoreceptors in the skin of man there are two sets of end organs, one responsive to cold and the other to heat.

The results obtained on the gills of *Unio* may be explained after analogy with the vertebrate heart, where the fastest beating chamber, the venous sinus, controls directly the rate of beat of the other chambers, which are inherently slower. In the same way the fastest beating cilia seem partially to determine the rate of adjacent, naturally slower cilia. This may be attributed to the stimulation of adjacent cells by the action current of their more active neighbors. Such a view is supported by work of R. S. Lillie (1914) on the swimming plates of ctenophores. In these animals successive waves of coordinated beating arise from the aboral end of a row of plates and pass down over the entire row. If, now, such a row with underlying tissue, be excised and placed in sea water, it will curl up so that the opposite ends are juxtaposed though no organic connection exists between them. Under these conditions Lillie maintains that the wave of beating, arising from the aboral end and passing down the row, jumps across the gap, and continues round and round in this way indefinitely. He explains this jump across the interruption as due to the stimulating effect of the action current of the swimming

plates of the oral end of the row on the adjacent plates of the aboral end. This explanation of conduction in ciliated epithelium would not, however, be in accord with the observation of Kraft that the excitatory effect of a stimulus may be transmitted through a region of quiescent cilia to excite other cilia beyond. This fact demands some other explanation of the mechanism of conduction, at least in the frog.

V.

CONCLUSIONS.

Evidence from several sources indicates that there is present in certain types of ciliated tissue a primitive form of conduction regulating the rate of beat of the cilia. (Kraft, 1890; Engelmann, 1898). A similar type of conduction has been observed by Parker (1910) in the sponge *Stylotella*. In the foregoing experiments a study was made of such conduction in the ciliated tissue of the gills of the clam *Unio*. Observations were made on the effects of temperature changes on the rate of beat of cilia adjacent to areas not themselves directly influenced. The following results were obtained.

1. The transmission through the gill of the effects of warmth applied locally is apparent through increased rate of ciliary beat on adjacent gill tissue in all directions from the region of application. Effects are not observed laterally at a distance greater than 9 to 11 mm. from the nearest edge of the stimulated area. The narrowness of the gill makes it impossible to determine the vertical limits of the transmission.

2. Effects of low temperatures are not observable beyond the limits of the region of direct application.

These results differ from those of Kraft in tissue from the frog's pharynx, where conduction was shown to take place only *down* a row of beating cilia. On the other hand they agree with his results in indicating that the effects of warmth only are transmitted.

The phenomenon might be explained by the stimulating effect of the action current of the directly excited cilia on the neighboring relatively quiet cilia. A similar explanation has been offered by Lillie for waves of coordinated beating in the rows of swimming plates of

ctenophores. Such an explanation, though in accord with the work on *Unio*, is inconsistent with certain of the observations of Kraft on the tissue from the frog's pharynx.

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ON THE IMPORTANCE OF MAINTAINING CERTAIN DIFFERENCES BETWEEN CELL SAP AND EXTERNAL MEDIUM.*

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The sap of *Valonia macrophysa* (collected at Bermuda) differs greatly in composition from the sea water in which the cells live, as is shown by Table I.[1]

TABLE I.

Composition of Valonia Sap and of Bermuda Sea Water.

Molecular proportions expressed as per cent of halides (Cl + Br).

	Cl+Br	Na	K	Ca	Mg	SO_4
Valonia sap	100	15.08	86.24	0.288	0	Trace?
Sea water	100	85.87	2.15	2.05	9.74	6.26

The sap contains about 1.4 parts per thousand of "organic matter."

The pH value of the sap is in the neighborhood of 5.9, while that of the sea water is in the neighborhood of 8.2.

The halide content of the sea water is about 0.6 M, while that of the sap (and likewise its freezing point depression) is a little greater.

The conductivity of the sap is noticeably higher than that of the sea water (about 20 per cent) as is to be expected, since the specific conductivity of KCl is greater than that of NaCl.

The sap is contained in a large central vacuole surrounded by protoplasm, which is in turn surrounded by a cell wall permeated with sea water. As is evident from the table, the inner and outer surfaces of the protoplasm are in contact with quite different media. Is it

* Contributions from the Bermuda Biological Station for Research, No. 148.

[1] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 225. The identification of this species is somewhat uncertain.

necessary for the life of the cell that these media should be different? What will happen if they are made identical? To answer this question, sap was collected by pricking live cells and quickly squeezing out the contents of the vacuole. The sap comes out as a perfectly clear liquid free from protoplasm. Most cells immersed in this sap lived but a short time (as a rule less than a week) while in sea water under the same conditions the majority live for several months.[2]

In view of the fact that the sap contains a little organic matter, it might seem possible that the rapid death of the cells was due in part to bacterial action. It was found that in sap which had been boiled and filtered the cells died as soon as in unboiled sap. To test the matter further an artificial sap free from organic matter was made by mixing 0.6 M KCl 172 cc. and 0.6 M NaCl 30 cc.: the pH values were brought to 6.0, 7.1, and 9.1.[3] In these solutions the cells lived less than 3 days.

The cells died promptly whether the normal difference in hydrogen ion concentration between sap and external solution was maintained or not; hence it is not the primary factor involved. It is, however, not negligible, since cells in sea water at pH 6 do not live as long as in normal sea water.

If death is due to a lack of balance in the proportion of salts, the toxic action should disappear on the addition of the salts needed to produce a balanced solution. Artificial sea water was made up by mixing the following (all 0.6 M): NaCl 1,000, $MgCl_2$ 78, $MgSO_4$ 38, KCl 22, $CaCl_2$ 20 cc.; this was brought to pH 8.1. Practically all the cells were alive at the end of 3 months,[4] when the experiment was discontinued. It is therefore evident that death is due to lack of balance and that the salts themselves contain no deleterious impurities (*e.g.*, copper) the effects of which could not be counteracted by adding other salts.

In order to test this idea further, calcium was added in various

[2] The cells were placed in finger-bowls covered with glass and kept away from direct sunlight.

[3] The pH value of the solutions was adjusted in all cases by adding HCl or NaOH.

[4] At the start the average temperature was about 25°C. and at the end about 19°C.

proportions to the artificial sap and the mixtures were brought to the pH values stated below (all the solutions were 0.6 M). To 202 cc. artificial sap (KCl 172 cc. + NaCl 30 cc.) were added $CaCl_2$ (*a*) 0.6 cc. (pH 6, 6.9, 7.9): (*b*) 2 cc. (pH 6.2, 6.9, 8.0): (*c*) 4 cc. (pH 6.1, 6.9, 8.1): (*d*) 6 cc. (pH 5.8, 7.0, 7.9): (*e*) 8 cc. (pH 6.0, 7.1, 7.9): (*f*) 10 cc. (pH 6.0, 7.0, 8.0). In these solutions all the cells died within 2 weeks.

It is therefore evident that the sap is not a balanced solution in the ordinary sense of the word nor can it be made so by the addition of calcium. In order to preserve the life of the cells the external medium must contain a larger proportion of sodium[5] than was used in the experiments just described. It was found that after a month in 0.6 M NaCl 1,000 cc. plus 0.6 M $CaCl_2$ 20 cc. (pH 8.1) half the cells were alive and in good condition whereas they died in a few hours in 0.6 M NaCl (pH 8.0).

The object of these experiments was not to determine the antagonistic action of different ions, nor to discover the precise mechanism of death, but to ascertain whether certain differences between the internal and external medium are necessary for the life of the cell. This seems to be the case beyond question, at least as far as the experiments have gone. If we could in any other way make the external and internal solutions identical, as by drawing the sap out of the cell and putting sea water in its place, it can hardly be doubted that such a drastic change in the composition of the sap would be disastrous.

We must therefore, in this case, conceive of the living substance as a very thin membrane stretched between two solutions of very different composition, and it appears that certain of these differences are necessary for the normal life of the cell.

The reason for this is not yet apparent. It is quite conceivable that it might be connected with differences in potential, but this does not seem to be the case. When a fine capillary tube filled with sap is thrust into the cell the wound at once closes up and the cell may live for several days and present a normal appearance. If we lead off from this tube, and also from the outside solution, to a Compton

[5] *Cf.* Osterhout, W. J. V., *Bot. Gaz.*, 1912, liv, 532.

electrometer we are unable to detect any difference of potential[6] greater than 1 or 2 millivolts. This result is somewhat unexpected since the hydrogen ion concentration of the sap is about a hundred times as great as that of the sea water. The cause of this lack of potential difference is under investigation. It is probably connected with the high concentration of salts.

It should be borne in mind that as yet we know very little about the activities of ions in mixtures and it is possible that a study of these activities may throw some light upon this question.

To what extent the conditions here described are paralleled in other cases must be left for future investigation to decide.[7] It is probably true of both plant and animal cells that differences exist between the internal and external solutions and that these differences are highly important.

SUMMARY.

A striking difference exists between the internal and external solution in the case of *Valonia macrophysa*. If this difference is abolished by placing cells in their own sap most of them quickly die.

There is some ground for believing that the maintenance of differences between the sap and the external medium is of importance for vital processes.

The sap of *Valonia macrophysa* is not a balanced solution in the ordinary sense and the question may be raised whether in general the interior of the cell requires a balanced solution in order to maintain life: or it may be that we must distinguish between internal and external balanced solutions.

[6] Before the measurement the cell was lifted out of the sea water and the surface carefully dried with filter paper. In some cases the cell was rinsed for a few seconds in distilled water before the application of filter paper: this made no material difference in the result. During the measurement the cell was suspended in air with its lower end in contact with sea water to which one calomel electrode was connected by a string moistened with sea water. The capillary tube inserted in the upper end of the cell was connected with the other calomel electrode by a string moistened with saturated KCl. Both calomel electrodes were filled with saturated KCl (saturated with calomel). Cells 1 to 2 inches long were employed in these measurements.

[7] Investigations of this nature are in progress. In applying these conceptions to smaller cells it should be borne in mind that sap obtained by crushing cells may be altered or contaminated during its preparation.

TEMPERATURE CHARACTERISTIC FOR HEART RHYTHM OF THE SILKWORM.

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I.

The frequency of heart beat as function of temperature has been determined in a number of animals. It has been abundantly demonstrated that in a qualitative sense the Q_{10} ratios are of the order of magnitude found in connection with chemical reactions, and that within the range of non-destructive temperatures the Q_{10} ratios typically decrease as the temperature rises. Beyond this, little or no analysis has been made. It has been pointed out (Crozier, 1924–25, *a*) that the equation of Arrhenius (1889), with its modern theoretic underpinning, may with profit be applied to vital phenomena. Such application was in a very general way made by Arrhenius himself (1915) and in sporadic instances has been urged by others (*e.g.*, Dhar, 1920; Matisse, 1921). But we are not so much interested in the mere application of an equation which describes the course of such data, as we are in the specific values of the thermal constant which it yields. Systematic use of this relationship for the synthesis of varied data has been illustrated in the case of respiratory oxidations (Crozier, 1924–25, *b*), and by means of a number of processes among arthropods in which the rate of central nervous discharge as influenced by temperature is probably a controlling factor; the latter provide concordant values of the constant (temperature characteristic) $\mu = 12{,}200$ calories (Crozier, 1924–25, *a*).

The validity of such synthesis, and its empirical justification as a method of functional analysis, may be further tested in the case of the heart rhythm. It was especially desired to complete a survey of heart beat frequencies as affected by temperature in order to discover

if in different phyla of animals the limiting mechanisms for frequency of heart rhythm should not turn out to be detectably different; or if in a given organism this mechanism might not vary under different circumstances. For this purpose we have measured with great care the heart beat frequency of the silkworm, and have critically examined previously published observations pertaining to the heart rates of vertebrates and invertebrates. It is intended subsequently to discuss this earlier material in detail. A fairly complete bibliographic survey is given in the monograph by Kanitz (1915), and the literature has been more recently summarized by Przibram (1923); to these books reference is made for older citations.

II.

The pulsating heart of the silkworm is clearly visible through the whitish translucent dorsal integument of the abdomen. We have employed full grown larvæ, within 2 days of the onset of pupation. The caterpillars were derived from a single mass of eggs, and were grown in the laboratory in large glass dishes.[1] There is considerable uniformity of the heart rate at constant temperature; undoubtedly this is due not merely to the common conditions of development for the different individuals, but in large measure to the relatively homogeneous character of a population of these highly inbred animals. In the latter respect the silkworm is particularly suitable as experimental material, for genetic uniformity of individuals must be expected to eliminate some sources of irregularity (*cf.* Crozier and Hubbs, 1924).

The larvæ were studied singly, the heart beat being timed while the animal was quietly feeding. Movements, aside from those of respiration and of the head and jaws in chewing, were practically absent. In cases where a momentary peristaltic body movement appeared, the reading was disregarded. At 15–13°C. the feeding motions ceased, rather abruptly, but the animals remained straight and quiet.

[1] The convenience of *Bombyx* larvæ as material for experimental work was brought to our notice by Dr. R. W. Glaser, of The Rockefeller Institute for Medical Research, Princeton, New Jersey, in connection with another matter. Our thanks are due Dr. Glaser for this suggestion, and for a supply of silkworm eggs. We are grateful to Mr. F. L. Campbell for his help in the care and feeding of the larvæ.

From nine to twenty beats were timed by watching the penultimate segment in which the heart is most distinct.

Temperature was controlled by immersing a beaker containing caterpillar and mulberry leaf in a large vessel of water maintained at the desired temperature. In a few cases temperature higher than that of the room was obtained in the air bath by using the radiant heat of an electric warmer. The beaker holding the larvæ was closed by a plate of cork. The temperature in the immediate vicinity of the larvæ was taken with a thermometer reading to 0.01°C.; correction was made for the temperature of the exposed stem.

Constancy of body temperature was assured by allowing at least 10 minutes for thermal adjustment to each new temperature. Before 5 minutes at a new temperature the heart rate becomes constant. When cooled below 9° departures from the chief line of Fig. 1 are evident after half an hour (or sooner); systole may be incomplete, and the rate then relatively high. On warming to 12–13° after half an hour spent at 5° or 6° the heart frequency is only slowly brought back to that normal for the higher temperature. Hysteresis of this sort is also the result of exposure to temperatures above 38°C.; it is not an uncommon phenomenon, and perhaps signifies physical alteration of the protoplasm which is only slowly reversible (*cf.* Loeb and Ewald, 1911). The measurements below 9° were made within half an hour of the larva's first being cooled from room temperature; observations made in this way are consistently reproducible.

The results with five individuals are given in Fig. 1; the logarithm of the heart rate (*i.e., 1 divided by time for one beat*) is plotted against the reciprocal of the absolute temperature. Each plotted point is the mean of three or more closely concordant measurements.

III.

The slope of the line[2] in Fig. 1 gives the value of the constant μ in the equation

$$\frac{K_2}{K_1} = e^{\frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}.$$

[2] It has been found by trial that in fitting data of the type plotted, as good an adjustment can be made by eye as can be made by least squares. And in fact, as ordinarily employed, the least squares procedure would require the assumption that the temperature measurements were devoid of variation (*cf.* Uhler, 1923).

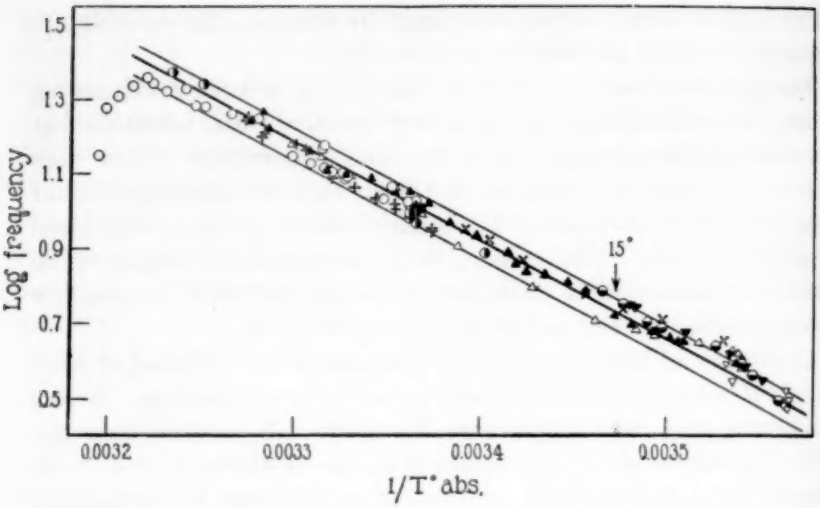

FIG. 1. Frequency of the heart beat in mature *Bombyx* larvæ of uniform antecedents and history. Each plotted point is the mean of a series of observations, the different individuals being distinguished by symbols. In the region 15–13°C. the frequency of pulsation is accelerated after a time, hence the irregularity in the curve. The readings for the lowest portion of the graph were obtained within the first 20 minutes exposure to the low temperature.

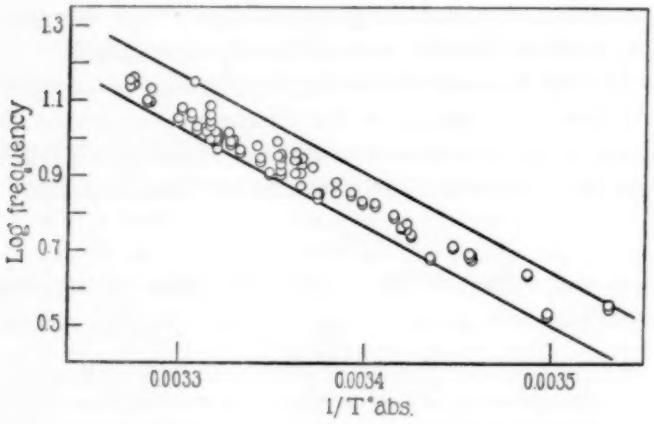

FIG. 2. Extreme variates in determinations of pulsation frequency of the hearts of three individuals.

K_2 and K_1 being measures of the velocity of the controlling process (*i.e.* of the heart beat frequency) at the respective temperatures. In this instance $\mu = 12{,}200$ calories.

It will be noticed (Fig. 1) that the variation of the mean results at any temperature is confined within definite limits These limits are $\pm$ 11.1 per cent of the mean. The character of this variation is important for the understanding of heart rate data. Fig. 2 contains a plot similar to that in Fig. 1, but giving the extreme high and the extreme low rates observed in consecutive series of observations with three individuals. The extent of fluctuation at constant temperature is less for each single individual than for the group, even though the different individuals are unusually similar in history and in fundamental (genetic) composition. This is adequate proof that the observed variation is real, organic, and not mere experimental error. The importance of this fact is realized when one attempts to analyze published data on heart frequencies. The confusion is often increased by the circumstance that on either side of a particular temperature the curve relating frequency to temperature may be totally different (*cf.* Crozier, 1924–25, *a*, *b*). For these reasons it is obviously quite insufficient to study merely the average frequency at each of several temperatures; it is necessary to know the frequencies at short intervals of temperature and also the law and extent of variation at constant temperature. The range of variation indicated in Fig. 2 is of slightly greater magnitude ($\pm$ 11.8 per cent of the mean) than that derived from the means of series of observations (Fig. 1), as statistically it should be. The information derived from Figs. 1 and 2 permits us to take the observations upon heart frequency in the silkworm as, in a way, a standard of comparison for similar but on the whole more fragmentary data pertaining to other animals.

IV.

The frequency of heart pulsation of the silkworm is controlled by a reaction having critical increment = 12,200 calories. The variation of pulsation rate at any temperature is at most $\pm$ 11.8 per cent of the mean for that temperature, in the homogeneous group of individuals studied. This figure may be taken to give the limits of

fluctuation in the effective concentration of the catalyst for the governing reaction.

An identical value of the temperature characteristic has previously been obtained for diverse phenomena, among arthropods, in which the rate of activity of central neurone groups is believed to be the determining factor (Crozier, 1924–25, *a*). An analogous mechanism presumably determines the contraction of the aliary musculature of the dorsal diaphragm of the silkworm, and hence the frequency of the heart beat. The concordance of the values of the temperature characteristics for these homologous activities strengthens the view that vital processes may submit to rational classification in this way.

SUMMARY.

The critical thermal increment for the reaction controlling the frequency of the heart beat in mature silkworms is 12,200 calories. This determination agrees quantitatively with the increment deduced for other activities of arthropods in which the rate of central nervous discharge is believed to be the controlling element.

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THE TEMPERATURE CHARACTERISTIC FOR PHARYNGEAL BREATHING RHYTHM OF THE FROG.

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I.

Published observations upon metabolism of frogs as a function of temperature suffice to indicate that a zone of temperature exists in the neighborhood of 15°C. which is a kind of critical temperature, in the sense that there the curve relating temperature to velocity of vital processes suffers modification. As one aspect of this condition there may appear the practical independence of the speed of gaseous exchange and the temperature, between 15° ± and 20° ± (Vernon, 1897; Jones, 1923). This independence has been interpreted to signify capacity of the frog to regulate its own metabolism, independently of environmental temperature, within a certain range. We shall point out that this conclusion may not be altogether necessary, or correct. For a number of other instances, including activities of organs and tissues of frogs, it has been shown that at about 15° there may occur a sharp change in the relationship between temperature and the velocities of protoplasmic activities (Crozier, 1924–25, *a*, *b;* Crozier and Pilz, 1924; Crozier and Federighi, 1924–25, *a;* Glaser, 1924–25), and that among the several expressions of such effects (Crozier 1924–25, *a;* Crozier and Stier, 1924–25) there may be found, at about 15°C. relatively enhanced variability in the biological result, or a zone of very slight average change in the temperature relation. Unpublished experiments with the ciliated epithelium of frog esophagus show a similar change in the curve of relation between ciliary activity and temperature. We desired to see, therefore, if the supposed autonomous control of the frog's metabolic processes within an interme-

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diate range of temperatures might not find its explanation in terms of such observations.

For this purpose, in part, we have studied the pharyngeal breathing rhythm. It will be shown that there does exist a small intermediate range of temperatures in which the frequency of the pharyngeal rhythm of frogs may seem independent of temperature; we believe that the explanation is in large part to be given through the conception of interconnected processes determining the breathing rhythm.

A further reason for the study of pharyngeal rhythm in the frog lies in the opportunity for partially testing one view as to the control of the activity of the medullary respiratory center. According to Winterstein (1911) and others, hydrogen ion may be the respiratory hormone. If the frequency of pharyngeal movements were determined through some event catalyzed by H^+, it might be expected that the critical thermal increment for this rhythm would correspond with that for known H^+-catalyzed processes (*i.e.*, about 20,000 calories). The experiments fail to support the notion of direct catalysis by H^+, and in this agree with (unpublished) experiments upon fishes; but beyond this they do not permit more than a suggestion as to the nature of the controlling process. The movements involved are pharyngeal movements, serving merely to renew air within the buccal cavity and during which the glottis is closed, not proper movements of lung ventilation (Baglioni, 1900; 1911), although presumably innervated from the respiratory center (for review of innervation, *cf.* Black, 1917). The flank movements, indicating lung filling, seem, however, to follow the same curve as do the pharyngeal movements—so far as our observations extend. The question may also be complicated by the rôle of skin respiration; in this respect the frog as a test object is perhaps inferior to the insects we have studied previously (Crozier and Stier, 1924–25).

II.

In measuring the pharyngeal rhythm of frogs (*Rana pipiens*) each individual, previously adapted during 4 to 35 days to ordinary room temperature, was placed in a 500 cc. bottle immersed in a large jar of water of which the temperature was under control. Through the totally immersed bottle was drawn a continuous current of mois-

tened air previously brought to thermostat temperature by passage through a coil of tubing in the water. A sensitive thermometer graduated to 0.1° had its bulb adjusted near the frog's head. In a few experiments the air was changed intermittently. If the thermostat be surrounded with yellow paper, feebly illuminated by indirect light, the frog maintains an almost constant position for hours. Movements of the pharyngeal floor are then easily observed through a small window in the jacket of the thermostat. The absence of spontaneous movements, thus largely insured by removing the likelihood of visual stimulation, is extremely important for significant measurements of respiratory frequency. The illumination, of itself, does not materially influence the pharyngeal rhythm (Crofts and Laurens, 1924).

The frequency of pharyngeal movements shows cyclic fluctuation, as previously noted for breathing movements of insects (Crozier and Stier, 1924–25). As a rule the shallow movements at the beginning of a breathing cycle are slower than the deeper movements at its close, leading up to lung inflation, but this correlation may be reversed. Some six to ten spaced observations were accordingly taken with each constant temperature, the time for ten movements being obtained at each observation. The plotted points are averages of such series. The temperature was changed by small steps. After each such change, at least 10 minutes was allowed for thermal adjustment. The immediate effect of even a slight change in temperature, whether of elevation or lowering, is to produce a brief but detectable acceleration in respiratory frequency. This temporary acceleration is probably due to thermal stimulation of the skin (Babák and Dýšek, 1912) (or nares?), and should give a delicate method for its investigation.

Each experiment consisted in obtaining a series of readings with a single animal over a convenient range of temperatures. Eighteen winter individuals, weighing 20 to 30 gm. each, were thus studied. Within two thermal regions respectively above and below 15° ± the effects of warming or of cooling are quickly reversible. But if one attempts to pass quickly from, say, 18° to 8°, complications may appear. In the light of experiments designed to test the mechanism of thermal adaptation it seems to us probable that these complications will prove useful in analyzing the basis of long-period adaptation to particular temperatures. Our purpose now, however, is merely to

obtain significant values of the *temperature characteristic* for pharyngeal rhythm, and discussion of adaptation is postponed.

III.

The result of a single typical experiment is given in Fig. 1. The possible working range is from about 6–28° or less. It is clear that in the vicinity of 15° there occurs a change in the effect of temperature. The expression of this change is somewhat different from that observed in a number of other cases (Crozier, 1924–25, *b*). For the latter, involving an unmistakable shift in the slope of the log $V - \frac{1}{T}$ line, the explanation was advanced that on either side of 15° ± a different link in a catenary chain of reactions controlling the end-result becomes the slow process determining the velocity of the whole. It should be clear that this type of explanation, for which there is indication of experimental support (Crozier and Stier, 1924–25), need not prevent the recognition of other kinds of temperature effects. Thus it might be supposed that the velocity of a given vital process is determined by the velocity of formation of a substance, X, but that within a particular range of temperatures the effective amount of this material could not be brought to exceed a certain maximum quantity. There is evidence for the occurrence of such cases, in which we may assume that the significant material, X, or a precursor of it, accumulates as formed in a surface, up to the point of saturation; unless physical change should occur in the structure of the surface, it may remain saturated with X over a wide range of temperature; and the velocity of the dependent process correspondingly remain sensibly constant.

While this conception may prove useful, it may not be necessary. The peculiar course of the curve in Fig. 1 may be due in part to that averaging of the effects of competing processes which is implicit in the taking of the observations. This is particularly true because the temperature 15° ± 1° is a region where, even in spite of careful shielding from visual and other disturbance, the frog tends to exhibit one or more spontaneous jumps, or sudden changes of position. For example, if the animal be very slowly cooled (0.1° per 10 minutes) from, say, 23°, it is motionless until approximately 15° is reached. By the time 14° is reached the frog is quiet again, and may so continue

for as much as 8 hours. This trigger effect is very curious, but we believe it comprehensible in terms of the theory already discussed (Crozier, 1924–25, *a*, *b;* Crozier and Federighi, 1924–25, *a;* Crozier and Stier, 1924–25). When muscular movements occur, the frequency

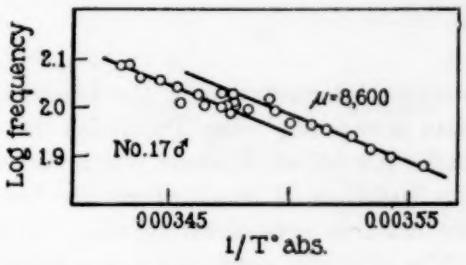

FIG. 1. Observations with Frog 17, illustrating characteristic break in the temperature effect. Each point is the mean of a series of observations at constant temperature.

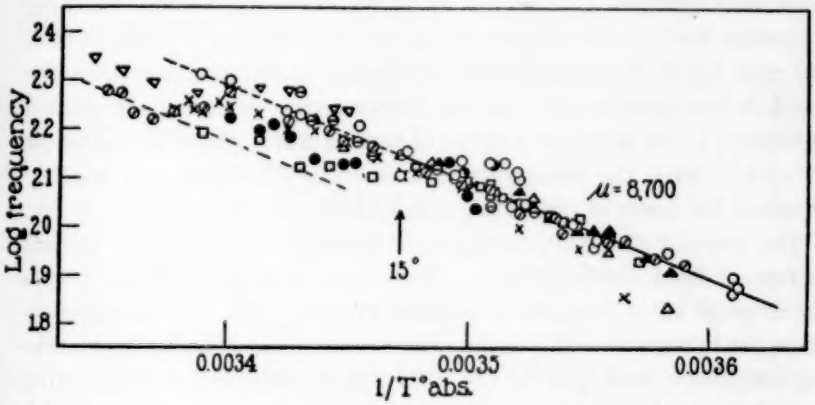

FIG. 2. Experiments with frogs not adjusted to laboratory temperature (*cf.* text); the data for one animal have been multiplied by 2.9 in order to bring them into the field of the remaining (unadjusted) observations.

of pharyngeal rhythm is increased. Consequently there appears a distinct bump on some curves such as are shown in Fig. 2.

It may be emphasized that if the breathing rhythm were to be studied from the usual standpoint of temperature coefficients (Q_{10} ratio), these types of irregularity could scarcely be dealt with, and

would indeed probably escape detection altogether. Effects of the sort illustrated in Fig. 1, and subsequent figures, are scarcely explicable by the assumption that the frog is able to control its body temperature, within a certain zone of external temperatures near 15°.

IV.

To obtain a working interpretation of the temperature effect a larger array of data is required. Fig. 2 contains the records from several frogs of similar size and antecedents, which were under laboratory conditions for less than $2\frac{1}{2}$ days—previously having been exposed to low temperatures approximating that out-of-doors (*i.e.*, about 10—15°). The plottings consistently demonstrate the occurrence of irregularity at about 15°. The temperature characteristic for the lower range of temperatures (6–15° ±) is $\mu = 8{,}700$. Fig. 3 contains similar records for frogs adapted for 4 to 39 days to ordinary laboratory temperature; here $\mu = 8{,}830$. The figures for breathing frequency are less concordant among the different individuals than is the case for those not adjusted to laboratory temperatures, but the break is less pronounced. In the former group, however, the critical increment is the same for a range of temperatures above 15° as below 13°, while with the non-adjusted animals the frequency is sensibly constant for zones of different extents above 13–15°.

The amount of variation makes it desirable to deduce the critical increment from combined data. The value thus secured agrees with the average value from the individual graphs. The individual differences are in part traceable to the extent of intrinsic variation in breathing frequency, and thus to the difficulty of obtaining a good fitting line when the slope (Figs. 2 and 3) is slight; the low rate of change with changing temperature, moreover, tends to magnify errors of timing.

A critical increment agreeing sufficiently well with that obtained for *Rana pipiens* has been deduced from the breathing rhythm of the bull-frog (*Rana catesbiana*). Two sets of observations are plotted in Fig. 4.

From the standpoint employed in previous papers this result is taken to mean that the frequency of breathing movements is governed by a reaction which controls the velocity of production of a deter-

mining substance. The critical increment characteristic of this reaction appears to belong among the group of those associated with energy-providing processes in protoplasm (Crozier, 1924–25, *b;* Crozier and Stier, 1924–25).

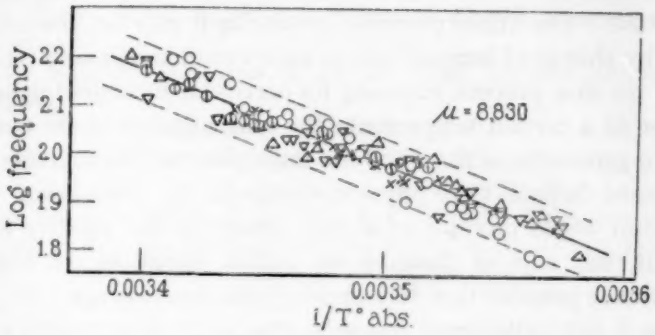

FIG. 3. Experiments with frogs adapted to laboratory temperature for 4 to 39 days. The averages from each animal are multiplied by a specific factor, to facilitate comparison.

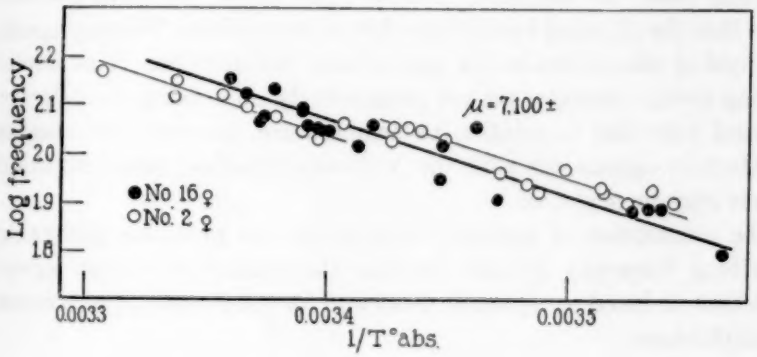

FIG. 4. Experiments with two bull-frogs (*Rana catesbiana*).

V.

To make the picture definite, let it be supposed that the frequency of the respiratory movements depends upon synaptic activities; for example, upon the frequency with which the synaptic galaxy in the respiratory center acts as an open valve, permitting escape of im-

pulses constantly generated within internunical neurones of the respiratory center. The open state of the valve is then taken to be a function of reactions occurring in the synapse. The velocity with which molecules of the effective substance are produced may conceivably be caused to differ in several ways on either side of a recognizably critical temperature. The linked processes producing it may be differentially affected by change of temperature to such extent that a new reaction becomes the slow process, imposing its particular increment upon the whole; or at a certain temperature the concentration of the mother-substance generating active catalytic molecules for this reaction may be decreased through some physical change in the protoplasm. The latter result would produce an abrupt change in the velocity of the end-result, but *without changing the critical increment* (*cf.* Fig. 1). It is of course possible that *both* these effects should occur simultaneously; or if not quite simultaneously, that their independent occurrences should contribute to irregularity in the vicinity of, say, 15° ±. Inspection of the plottings in Figs. 2 and 3 shows that in some cases the frequency of movements at temperatures above 15° falls consistently *above* the line derived from observations below 15°, rather than that the physical basis of the shift is irreversible. We may speak of physical alterations in the protoplasm (surface films ?) as determining abrupt changes, yet not progressively influencing the velocity of vital activities in relation to temperature, because one obtains satisfactory agreements with the Arrhenius equation; hence diffusion effects may be neglected.

The assumption of synaptic location for the processes governing breathing frequency is made because the critical increment agrees with that deduced for synaptic delay and for some related phenomena in amphibians.

SUMMARY.

The frequency of the pharyngeal respiratory rhythm of frogs exhibits a critical thermal increment $\mu = 8{,}800$ calories. At about 15° irregularities are apparent, which may be reduced by continued adaptation to room conditions. The frequency depends upon a process possibly synaptic in locus and apparently belonging among the

group of respiratory reactions. Its temperature characteristic sharply separates this process from those reactions known to be catalyzed by H ion.

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